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**Cellular mechanisms for the control of
glucocorticoid metabolism by 11-beta
hydroxysteroid dehydrogenase in the human
ovary**

A thesis submitted by

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2005

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For my parents
Yvonne and John

Abstract

The aims of this thesis were to: (i) determine the regulatory properties of human follicular fluid (FF) on the activities and expression of 11 β HSD, (ii) investigate the effects of progesterone on 11 β HSD activity and expression (iii) investigate the effects of prostaglandins on basal and endocrine-stimulated 11 β HSD activity and expression (iv) to predict the topology and tertiary structure of 11 β HSD1. Human FF contains hydrophilic and hydrophobic compounds that stimulate and inhibit, respectively, the NADP(H)-dependent activities of 11 β HSD1. The predominant lipid inhibitors of 11 β HSD1 in FF are unlikely to be steroids or prostaglandins, and the effects of the FF components on enzyme activities occur without changes in 11 β HSD1 protein expression. Inhibition of progesterone production by aminoglutethimide increased 11 β HSD activities, without affecting 11 β HSD1 protein expression in human granulosa-lutein cells. Pharmacological inhibitors of prostaglandin-H synthase (PGHS)-2 (meclofenamic acid (MA) and NS-398) decreased cortisol-cortisone inter-conversion by up to 50%, suggesting that prostaglandins may stimulate ovarian 11 β HSD activities in a paracrine/autocrine manner. Furthermore, PGHS-2 inhibitors prevented human chorionic gonadotrophin (hCG) and IL-1 β from stimulating 11 β HSD activities in human granulosa-lutein cells, implicating eicosanoids in the stimulation of ovarian 11 β HSD by gonadotrophins and cytokines. Since MA and NS-398 had no effect on 11 β HSD1 protein expression, the stimulation of 11 β HSD activities by eicosanoids occurs at the post-translational level. Finally, the structure of 11 β HSD1 protein was modelled to develop molecular mechanisms by which prostaglandins, gonadotrophins and cytokines might regulate enzyme activities. Although the primary sequence of 11 β HSD1, which is highly conserved across 10 mammalian species, contains several consensus phosphorylation sites for serine/threonine and tyrosine kinases, models of 11 β HSD1 topology predict that most phosphorylation sites would lie in the lumen of the endoplasmic reticulum. 11 β HSD1 includes hydrophobic α -helices neighbouring the active site, which may provide regions for the allosteric control of glucocorticoid metabolism by progesterone and eicosanoids in ovarian cells.

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Table of Contents

Abstract.....	3
Acknowledgements	4
List of Figures	10
List of Tables.....	15
Publications	16
Papers.....	16
Abstracts.....	17
Abbreviations	19
Chapter One: General Introduction	24
Chapter 1- General Introduction.....	25
1.1 The Human Ovary.....	25
1.1.1 Formation of the human ovary.....	25
1.1.2 The Ovarian cycle: Folliculogenesis, Ovulation and Luteinisation.....	26
1.2 Ovarian Steroidogenesis.....	33
1.2.1 Cholesterol uptake and transport.....	33
1.2.2 Ovarian Steroidogenesis.....	36
1.3 Eicosanoids and Ovarian function	39
1.3.1 Prostaglandin Biosynthesis.....	39
1.3.2 Lipoxygenase	42
1.3.3 Epoxygenase	43
1.3.4 Role of Eicosanoids in Ovulation	43
1.3.3 Role of eicosanoids in CL function.....	46
1.4 Role of LH in Ovarian function.....	48
1.5 Role of Interleukin-1 in Ovarian function.....	50
1.6 Cortisol Metabolism.....	52
1.6.1 Synthesis of glucocorticoids	52
1.6.2 Circulation of glucocorticoids.....	54
1.6.3 Action of Glucocorticoids	54
1.6.4 Glucocorticoid-metabolising enzymes	55
1.7 11 β HSD.....	57
1.7.1. 11 β HSD1	58
1.7.2 11 β HSD2	60

1.7.3 11 β HSD3	62
1.7.4 11 β HSD enzymes and Ovarian function	62
1.8 Project aims and justification	71
Chapter 2- Materials and Methods	74
2.1 Chemicals and Reagents.....	74
2.2 Collection and storage of follicular aspirates	76
2.2.1 Patient samples.....	76
2.2.2 Isolation of human granulosa cells.....	77
2.3 Radioimmunoassays.....	78
2.3.1 Progesterone RIA	78
2.3.2 PGE ₂ RIA.....	80
2.3.3 PGF _{2α} RIA	82
2.4 11 β HSD Assay	84
2.5 Reverse Phase C18 column chromatography	86
2.6 Assessment of 11 β HSD enzyme modulators in FF	87
2.7 Cell isolation and protein assay for Western blot.....	88
2.8 SDS-PAGE	89
2.9 Western Blot	90
2.9.1 Preparation for transfer of proteins from SDS-PAGE gel to polyvinylidene difluoride (PVDF) membrane.....	90
2.9.2 Blocking of the membrane.....	91
2.9.3 11 β HSD1 primary antibody probing.....	91
2.9.4 Secondary antibody probing	91
2.9.5 Developing of the blot	92
2.9.6 Stripping the membrane of bound antibody	92
2.9.7 GAPDH re-probing	92
2.9.8 Quantification of protein expression	93
2.10 Statistical analyses	93
Chapter Three: Effect of human follicular fluid on the activity and expression of 11 β HSD1	95
Chapter 3: Effect of human follicular fluid on the activity and expression of 11 β HSD1	96

3.1 Background.....	96
3.2 Experimental protocol.....	97
3.3 Results	99
3.3.1 Effects of human FF on NADP(H)-dependent 11 β HSD activities.....	99
3.3.2 Effects of human FF on NAD ⁺ -dependent cortisol oxidation	100
3.3.3. Elution profiles of ³ H lipids from C18 cartridges	101
3.3.4 Effect of human FF fractions on 11 β HSD1 expression in hGL cells	101
3.4 Discussion.....	101
Chapter Four: The autocrine/paracrine effects of progesterone on the activities and expression of 11 β HSD1 in human granulosa-lutein cells.....	114
Chapter 4-The autocrine/paracrine effects of progesterone on the activities and expression of 11 β HSD1 in human granulosa-lutein cells.....	115
4.1 Introduction	115
4.2 Experimental Design.....	116
4.3 Results	118
4.3.1 Concentration-dependent effects of exposure to AG on progesterone production in human granulosa-lutein cells	118
4.3.2 Concentration dependent effects of AG on 11 β HSD activities in human granulosa-lutein cells	118
4.3.3 Effect of AG on 11 β HSD1 expression in human granulosa-lutein cells	123
4.4 Discussion.....	123
Chapter Five: Paracrine roles for eicosanoids in determining the basal activities and protein expression of 11 β HSD1 in human granulosa-lutein cells.....	130
Chapter 5: Paracrine roles for eicosanoids in determining the basal activities and protein expression of 11 β HSD1 in human granulosa-lutein cells.....	131
5.1 Introduction	131
5.2 Experimental design.....	132
5.2.1 Effects of MA and NS-398 on prostaglandin production, 11 β HSD activities and 11 β HSD1 expression	132
5.2.2 Effects of exogenous prostaglandins on cortisol oxidation	134
5.3 Results	134
5.3.1. Effect of MA on basal prostaglandin production.....	134

5.3.2. Effect of MA on 11 β HSD activities in human granulosa-lutein cells ..	135
5.3.3. Effects of MA on 11 β HSD1 protein expression.....	136
5.3.4. Effects of NS-398 on basal prostaglandin production.....	136
5.3.5 Effect of NS-398 on 11 β HSD activities in human granulosa-lutein cells	136
5.3.6 Effect of NS-398 on 11 β HSD1 protein expression.....	137
5.3.7 Effect of exogenous prostaglandins on cortisol oxidation.....	137
5.4. Discussion.....	152
Chapter Six: Role of prostaglandins in mediating the effects of hCG and IL-1 β on the activity and expression of 11 β HSD1 in human granulosa-lutein cells.....	159
Chapter 6: Role of prostaglandins in mediating the effects of hCG and IL-1 β on the activity and expression of 11 β HSD1 in human granulosa-lutein cells.....	160
6.1 Introduction	160
6.2 Experimental design.....	161
6.2.1 Acute and chronic effects of hCG and IL-1 β on prostaglandin production and 11 β HSD activities	161
6.2.2 Concentration-dependent effects of IL-1 β and hCG on prostaglandin synthesis and 11 β HSD1 activities	162
6.2.3 Effects of hCG and IL-1 β on 11 β HSD1 protein expression	162
6.3 Results.....	163
6.3.1 Effects of hCG and IL-1 β on PGE ₂ and PGF _{2α} production.....	163
6.3.2 Acute effects of hCG and IL-1 β on 11 β HSD activities	164
6.3.4 Concentration-dependent effects of hCG on PGE ₂ and PGF _{2α} production	166
6.3.5 Concentration-dependent effects of IL-1 β on PGE ₂ and PGF _{2α} production	166
6.3.6 Concentration-dependent effects of hCG on 11 β HSD activities: effects of preferential-PGHS2 inhibitors	166
6.3.7 Concentration-dependent effects of IL-1 β on 11 β HSD activities: effects of preferential PGHS2 inhibitors	167
6.3.8 Effect of hCG and IL-1 β on 11 β HSD1 protein expression.....	195
6.4 Discussion.....	195

Chapter Seven: Elucidation of structural topology, regulatory domains, and tertiary structure of 11 β HSD1	201
Chapter 7: Elucidation of structural topology, regulatory domains, and tertiary structure of 11 β HSD1	202
7.1 Introduction	202
7.2 Experimental Design	205
7.2.1 Primary sequence alignment	205
7.2.2 Functional site characterisation of the 11 β HSD1 primary sequence	206
7.2.3 Secondary structure predictions	206
7.2.4 Localisation of TM domains	206
7.2.5. Tertiary structure prediction- Fold recognition	207
7.3 Results	208
7.3.1. Alignment of 11 β HSD1 primary sequences	208
7.3.2. Alignment of bovine 11 β HSD1, 11 β HSD2 and 11 β HSD3 primary sequences	208
7.3.2 Analysis of functional sites and regulatory regions in all sequences aligned of 11 β HSD1	208
7.3.3. Secondary Structure prediction for human 11 β HSD1	209
7.3.4. Topology predictions for 11 β HSD1	209
7.3.5. Tertiary structure models	210
7.3.6 Protein fold predictions for the luminal domain of human 11 β HSD1 ..	237
7.4 Discussion	237
Chapter Eight: General discussion	242
Chapter 8: General Discussion	243
References	256
References	257
Appendix I	295
Appendix I	296
Appendix II	300
Appendix II	301
Appendix III	307
Appendix IV	311

List of Figures

Figure 1.1	Principle pathways of ovarian steroidogenesis	37
Figure 1.2	Principle pathways of glucocorticoid biosynthesis	53
Figure 1.3	Mechanism by which cortisol and cortisone are metabolised in target tissues and the liver for excretion	56
Figure 1.4	Inter-conversion of cortisol and cortisone by isoforms of the enzyme 11 β HSD	59
Figure 3.1	Effect of compounds in human FF eluted by reverse-phase C18 chromatography and stepwise methanol gradient fractionation on NADP ⁺ -dependent oxidation of 100nM cortisol over 1 hours in a rat kidney homogenate bioassay	102
Figure 3.2	Effect of compounds in human FF eluted by reverse-phase C18 chromatography and stepwise methanol gradient fractionation on NADPH-dependent reduction of 100nM cortisone over 1 hours in a rat kidney homogenate bioassay	103
Figure 3.3	Effect of compounds in human FF before and after incubation with dextran coated charcoal.	104
Figure 3.4	Effect of compounds in human FF eluted by reverse-phase C18 chromatography and stepwise methanol gradient fractionation on NAD ⁺ -dependent reduction of 100nM cortisol over 1 hours in a rat kidney homogenate bioassay	105
Figure 3.5	Elution profiles of exogenously added prostaglandins and steroids in human FF by reverse-phase C18 chromatography and stepwise methanol gradient fractionation	106
Figure 3.6	Representative Western blot of effect of whole non-fractionated hFF, and those fractions of human FF eluted at 0% (v/v) and 80% (v/v) methanol on expression of 11 β HSD1 (and GAPDH) in human granulosa-lutein cells	107
Figure 4.1	Concentration-dependent effects of treatment with AG on progesterone production over 4 and 24 hours by human granulosa-lutein cells on day 3 of culture	119
Figure 4.2	Concentration-dependent effects of AG on 11 β HSD activities	120

	over 4 hours on day 3 of culture in human granulosa-lutein cells	
Figure 4.3	Concentration dependent effects of AG on 11 β HSD activities over 24 hours on day 3 of culture in human granulosa-lutein cells	121
Figure 4.4	Effects of AG on 11 β HSD1 protein	122
Figure 5.1A	Concentration-dependent effects of treatment with MA for 4 hours on PGE ₂ and PGF _{2α} production in human granulosa-lutein cells on day 3 of culture	138
Figure 5.1B	Concentration-dependent effects of treatment with MA for 24 hours on PGE ₂ and PGF _{2α} production in human granulosa-lutein cells on day 3 of culture	139
Figure 5.2A	Concentration-dependent effects of treatment with MA for 4 hours on 11 β HSD activities in human granulosa-lutein cells on day 3 of culture	140
Figure 5.2B	Concentration-dependent effects of treatment with MA for 24 hours on 11 β HSD activities in human granulosa-lutein cells on day 3 of culture	141
Figure 5.3	Effect of MA on 11 β HSD1 protein	142
Figure 5.4A	Concentration-dependent effects of treatment with NS-398 for 4 hours on PGE ₂ and PGF _{2α} production in human granulosa-lutein cells on day 3 of culture	143
Figure 5.4B	Concentration-dependent effects of treatment with NS-398 for 24 hours on PGE ₂ and PGF _{2α} production in human granulosa-lutein cells on day 3 of culture	144
Figure 5.5A	Concentration-dependent effects of treatment with NS-389 for 4 hours on 11 β HSD activities in human granulosa-lutein cells on day 3 of culture	145
Figure 5.5B	Concentration-dependent effects of NS-389 on 11 β HSD activities in human granulosa-lutein cells on day 3 of culture over 24 hours	146
Figure 5.6	Effect of NS-398 on 11 β HSD1 protein expression	147
Figure 5.7	Effect of with 100 μ M MA \pm 1 μ M PGD ₂ , PGE ₂ , PGF _{2α} and PGI ₂	148

	on cortisol oxidation over 4 hours in hGL cells, on day 3 of culture	
Figure 5.8A	Effect of with 100μM MA ± 10μM PGD ₂ , PGE ₂ , PGF _{2α} and PGI ₂ on cortisol oxidation over 4 hours in hGL cells, on day 3 of culture in the absence of AG	149
Figure 5.8B	Effect of with 100μM MA ± 10μM PGD ₂ , PGE ₂ , PGF _{2α} and PGI ₂ on cortisol oxidation over 4 hours in hGL cells, on day 3 of culture in the presence of AG	150
Figure 5.9	Effect of with 100μM MA ± 10μM PGD ₂ , PGE ₂ , PGF _{2α} and PGI ₂ on cortisol oxidation over 4 hours in hGL cells, on day 3 of culture	151
Figure 5.10	Structures of Meclofenamic acid and NS-398	153
Figure 6.1A	Effects of hCG and IL-1β on PGE ₂ production over 4 hours in the absence of AG	168
Figure 6.1B	Effects of hCG and IL-1β on PGF _{2α} production over 4 hours in the absence of AG	169
Figure 6.2A	Effects of hCG and IL-1β on PGE ₂ production over 4 hours in the presence of AG	170
Figure 6.2B	Effects of hCG and IL-1β on PGF _{2α} production over 4 hours in the presence of AG	171
Figure 6.3A	Effects of hCG and IL-1β on PGE ₂ production over 24 hours in the absence of AG	172
Figure 6.3B	Effects of hCG and IL-1β on PGF _{2α} production over 24 hours in the absence of AG	173
Figure 6.4A	Effects of hCG and IL-1β on PGE ₂ production over 24 hours in the presence of AG	174
Figure 6.4B	Effects of hCG and IL-1β on PGF _{2α} production over 24 hours in the presence of AG	175
Figure 6.5A	Effects of hCG and IL-1β on cortisol oxidation over 4 hours in the absence of AG	176
Figure 6.5B	Effects of hCG and IL-1β on cortisone reduction over 4 hours in the absence of AG	177

Figure 6.6A	Effects of hCG and IL-1 β on cortisol oxidation over 4 hours in the presence of AG	178
Figure 6.6B	Effects of hCG and IL-1 β on cortisone reduction over 4 hours in the presence of AG	179
Figure 6.7A	Effects of hCG and IL-1 β on cortisol oxidation over 24 hours in the absence of AG	180
Figure 6.7B	Effects of hCG and IL-1 β on cortisone reduction over 24 hours in the absence of AG	181
Figure 6.8A	Effects of hCG and IL-1 β on cortisol oxidation over 24 hours in the presence of AG	182
Figure 6.8B	Effects of hCG and IL-1 β on cortisone reduction over 24 hours in the presence of AG	183
Figure 6.9A	Concentration dependent effects of hCG on PGE ₂ over 24 hours in the presence of AG	184
Figure 6.9B	Concentration dependent effects of hCG on PGF _{2α} over 24 hours in the presence of AG	185
Figure 6.10A	Concentration dependent effects of IL-1 β on PGE ₂ over 24 hours in the presence of AG	186
Figure 6.10B	Concentration dependent effects of IL-1 β on PGF _{2α} over 24 hours in the presence of AG	187
Figure 6.11A	Concentration dependent effects of hCG on cortisol oxidation over 24 hours in the presence of AG	188
Figure 6.11B	Concentration dependent effects of hCG on cortisone reduction over 24 hours in the presence of AG	189
Figure 6.12	Concentration dependent effects of hCG on cortisol oxidation over 24 hours in the presence of AG	190
Figure 6.13A	Concentration dependent effects of IL-1 β on cortisol oxidation over 24 hours in the presence of AG	191
Figure 6.13B	Concentration dependent effects of IL-1 β on cortisone reduction over 24 hours in the presence of AG	192
Figure 6.14	Concentration dependent effects of IL-1 β on cortisol oxidation over 24 hours in the presence of AG	193

Figure 6.15	Effect of hCG and IL-1 β on 11 β HSD1 protein expression of 11 β HSD1 standardised to GAPDH protein expression in the presence of AG	194
Figure 7.1A	Visual alignment of primary sequences of 11 β HSD1 from other mammalian species to human 11 β HSD1	214
Figure 7.1B	Primary sequence alignment of 11 β HSD1 from different mammalian species using CLUSTALW database and subsequent alignment to human 11 β HSD1 in order of evolutionary divergence	216
Figure 7.2	Primary sequence alignment of bovine 11 β HSD1, 11 β HSD2 and 11 β HSD3 using CLUSTALW database	218
Figure 7.3	Predicted conserved regulatory sites of 11 β HSD1	219
Figure 7.4	Secondary structure prediction for human 11 β HSD1 generated by PsiPred based on PsiBLAST scores when compared to other proteins within the PsiPred database	222
Figure 7.5	Hydropathy plot of aligned sequences for mammalian 11 β HSD1 enzymes	224
Figure 7.6	Hydropathy plot and transmembrane domain predictions for human 11 β HSD1 using TMAP, TopPred-KD, TopPred-GES, MEMSAT2 and TMHMM topology prediction methods	225
Figure 7.7A	Model 1 of secondary structure and topology within the ER membrane of human 11 β HSD1	226
Figure 7.7B	Model 2 of secondary structure and topology within the ER membrane of human 11 β HSD1	227
Figure 7.7C	Model 3 of secondary structure and topology within the ER membrane of human 11 β HSD1	228
Figure 7.8	Three- dimensional structures for the candidate structural homologues of human 11 β HSD1	229
Figure 8.1	Steroidogenic pathways utilising NADP(H) in human granulosa-lutein cells	253
Figure 8.2	Proposed mechanism by which prostaglandins and progesterone regulate 11HSD activities	255

List of Tables

Table 1.1	Regulatory compounds of 11 β HSD enzyme isoforms	67
Table 7.1	Table outlining homology to human 11 β HSD1 primary sequence comprised of homology to whole sequence, N terminus before the catalytic region and C terminus following the catalytic region	233
Table 7.2	Summary of essential criteria to assess the functionality of each proposed model for the topology of human 11 β HSD1	234
Table 7.3	Summary of mGenTHREADER tertiary structure homology	235

Publications

Papers

LM Thurston, DP Norgate, **KC Jonas**, C Chandras, HJ Kloosterboer, BA Cooke & AE Michael (2002) Ovarian modulators of 11β hydroxysteroid dehydrogenase (11β -HSD) activity in follicular fluid from gonadotrophin-stimulated assisted conception cycles. *Reproduction* 124:801-812

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KC Jonas, AE Michael (in preparation) Predictions of 11β Hydroxysteroid dehydrogenase type 1 topology and tertiary structure: an *in silico* approach

TM-M Tan, LM Thurston, **KC Jonas**, CW Perrett, MP Van der Pump, PJ Hardiman & AE Michael (in preparation) Ghrelin in human follicular fluid: paracrine stimulus to cortisol-cortisone inter-conversion by 11 β -hydroxysteroid dehydrogenase (11 β HSD)

LM Thurston, A Khan, **KC Jonas**, DRE Abayasekara & AE Michael (in preparation) Effects of hypoxia on 11 β -hydroxysteroid dehydrogenase (11 β -HSD) enzymes in human and bovine ovarian cells: further evidence for the existence of a novel 11beta-HSD isoform

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KC Jonas, H Vakharia, S Wylie, LM Thurston, AE Michael (2004) Effects of ovarian modulators on 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1) expression in human granulosa lutein cells. *12th International Congress of Endocrinology, Lisbon, Portugal, 31st August-4th September 2004*

Abbreviations

[I ¹²⁵]	radioactive isotope iodine 125
11 β -DH	11 β dehydrogenase
11 β HSD1	11 β hydroxysteroid dehydrogenase type 1
11 β HSD2	11 β hydroxysteroid dehydrogenase type 2
11-KSR	11-ketosteroid reductase
17 β HSD	17 β -hydroxysteroid dehydrogenase
3 β HSD	3 β -hydroxysteroid dehydrogenase
AA	arachidonic acid
AC	adenylyl cyclase
ACTH	adrenocorticotrophic hormone
AG	aminoglutethimide
ANOVA	analysis of variance
ATP	adenosine tri-phosphate
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
cAMP	cyclic adenosine 3'5' mono-phosphate
CBG	corticosteroid binding globulin
cDNA	complementary DNA
CL	corpus luteum
COX	cyclo-oxygenase
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium

DMEM:Ham's F12	Dulbecco's modified Eagles medium: Ham's F ₁₂
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EP	PGE ₂ receptor
EP1	PGE ₂ receptor type 1
EP2	PGE ₂ receptor type 2
EP3	PGE ₂ receptor type 3
EP4	PGE ₂ receptor type 4
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide
FSH	follicle stimulating hormone
GH	growth hormone
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
hCG	human chorionic gonadotrophin
HMG CoA	hydroxymethyl glutaryl coenzyme A
hOSE	human ovarian surface epithelial cells
hsp90	heat shock protein 90
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL-1 β	interleukin 1-beta
IVF	<i>in vitro</i> fertilisation
IVF-ET	<i>in vitro</i> fertilisation-embryo transfer
K _m	Michelis-Menton enzyme rate constant

LBD	ligand binding domain
LTB ₄	leukotriene B ₄
LH	luteinising hormone
LHR	luteinising hormone receptor
MA	meclofenamic acid
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NAD ⁺	nicotinamide adenine dinucleotide
NADP ⁺	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NDGA	nordihydroguaiaretic acid
NSB	non-specific binding
PBR	peripheral benzodiazepene receptor
PGDH	prostaglandin dehydrogenase
PGE ₂	prostaglandin E ₂
PGF _{2α}	prostaglandin F _{2α}
PGHS	prostaglandin H synthase
PGHS-1	prostaglandin H synthase type 1
PGHS-2	prostaglandin H synthase type 2
PKA	protein kinase A
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PLD	phospholipase D

PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
PVDF	polyvinylidene difluoride
rRNA	ribosomal ribonucleic acid
SEM	standard error of the mean
<i>SRY</i>	sex-determining region of the Y chromosome
StAR	steroidogenic acute regulatory protein
TGF β	transforming growth factor β
TLC	thin layer chromatography
TNF α	tumour necrosis factor α
TX	thromboxane
v/v	volume per volume
VEGF	vascular endothelial growth factor
w/v	Weight per volume

‘Research is what I’m doing when I don’t know what it is I’m doing’

Wernher Von Braun (1912-1977)

Chapter One: General Introduction

Chapter 1- General Introduction

Approximately 1 in 7 couples of reproductive age are infertile in the UK [Templeton *et al.*, 1991] and will require assisted reproductive technology in order to conceive a child. It is therefore essential to establish the fundamental mechanisms controlling ovarian physiology with a view to advancing reproductive technologies. The investigations reported in this thesis were designed to enhance our understanding of the regulatory mechanisms controlling ovarian glucocorticoid metabolism at a cellular level.

1.1 The Human Ovary

1.1.1 Formation of the human ovary

Until the 6th week of human embryo development, the male and female gonads are identical. It is at this stage of human foetal development that the Y chromosome determination of gonadal sex occurs through the expression of the *SRY* gene, which initiates Sertoli cell development and proliferation of male sex cords. The female gonads do not express the *SRY* gene and so remain indifferent to its effects. In the absence of *SRY* the Mullerian ducts differentiate into the Fallopian tubes/oviducts, and the external female genitalia form. About the time of the second trimester, the ovary and ovarian primordial follicles develop, with primordial follicle numbers equalling approximately 6 million. Mitotic division of oogonia ceases before birth, and under the control of the meiotic initiation factor, the oogonia undergo the first meiotic division to become primary oocytes. Oogenesis is arrested at prophase I, at the dictyate stage of the first meiotic division. At birth the human ovaries contain

approximately 2 million primordial follicles, which by puberty decreases to approximately 500,000 primordial follicles [Johnson and Everitt 2000]. Recent studies by Johnson *et al.* [2004] have challenged the model that there is a fixed pool of primordial follicles at birth, and have presented data in adult mice to suggest that germ stem cells are able to regenerate themselves in adult life.

1.1.2 The Ovarian cycle: Folliculogenesis, Ovulation and Luteinisation

Before puberty, the ovary is active, with follicles growing and undergoing atresia. At puberty, the process of folliculogenesis activates ovarian follicles to begin further development leading to ovulation of the oocyte. Each ovarian cycle can be split into 3 distinct phases, the follicular phase, the ovulatory phase, and the luteal phase. This cyclic pattern is controlled by interactions between the hypothalamus, (through the release of gonadotrophin-releasing hormone (GnRH)), anterior pituitary, (through the release of gonadotrophins under the control of GnRH [Fink, 1979]), and the ovaries [reviewed by McGee and Hsueh 2000].

1.1.2.1 The Follicular Phase

Primordial follicles are characterised as containing an oocyte surrounded by a flattened layer of granulosa cells. As the follicle develops, the granulosa cells start to divide and undergo morphological changes forming a cuboidal phenotype. At this stage the follicle is referred to as a primary follicle. The initial recruitment of primordial follicles and subsequent growth to the pre-antral stage is independent of circulating gonadotrophins. Resting follicles are thought to be under the control of inhibitory and stimulatory intra-ovarian factors that are thought to suppress or

initiate primordial follicle growth respectively. The mechanism of recruitment of primordial follicles remains unclear. However, the kit ligand has been implicated in the initial recruitment and formation of the primary follicle [Yoshida *et al.*, 1997]. Growth differentiation factor-9 (GDF-9), a peptide secreted by the oocyte, has also been implicated in primary follicle development. Follicles lacking GDF-9 have been shown not to proceed beyond the primary follicle stage [Aaltonen *et al.*, 1999].

Development of the primary follicle into the secondary follicle involves the growth of the oocyte, and further cell growth and differentiation. The undifferentiated cells surrounding the granulosa cells differentiate into thecal cells. These cells condense around the follicle forming 2 layers, the theca interna and the theca externa, which are separated from the granulosa cells by a basement membrane. The vascular supply infiltrates the thecal cell layer, but is excluded from the granulosa cells.

Granulosa-oocyte communication has been shown to be essential for oocyte development. Gap junctions exist between granulosa cells and the oocyte to mediate this communication. For example, connexin 37 is an important gap junction protein, essential for oocyte development [Simon *et al.*, 1997]. Paracrine communications between granulosa cells and the surrounding theca cell layers have also been shown to be important for the growth of pre-antral follicles. Recent studies have implicated fibroblast growth factor-7 as a paracrine factor secreted from theca cells that enhances pre-antral follicular growth [McGee *et al.*, 1999]. As granulosa cells proliferate a viscous liquid forms between the cells in droplet form. These droplets coalesce to form a single fluid-filled follicular antrum. At this stage the follicle can be described as an early antral follicle.

The gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH), are important trophic factors that control the proliferation, survival and recruitment of antral follicles. FSH is important for early antral follicle development, inducing expression of steroidogenic genes such as aromatase (*CYP19*), P450 cholesterol side chain cleavage (P450_{csc}, *CYP11A*) and the expression of LH receptors. Moreover, treatment of early antral follicles with FSH has been shown to prevent the onset of follicular atresia through apoptosis [Chun *et al.*, 1996]. Oestrogens are important anti-apoptotic signals in early antral development, the production of which is FSH dependent.

The 2-cell, 2-gonadotrophin model of oestradiol production reveals a functional synergy between LH and FSH. LH stimulates the synthesis of androstenedione in theca cells, forming the substrate for granulosa cell oestradiol synthesis. FSH stimulates aromatisation of androgens in granulosa cells to form oestrogens. The actions of FSH on granulosa cells are further enhanced by insulin-like growth factor-I (IGF-I) [Richards *et al.*, 2002] (this shall be covered more extensively in section 1.2).

The action of LH on the production of theca cell androgens can be mimicked by several paracrine factors. Members of the IGF subtype and transforming growth factor- β (TGF β) superfamily have been shown to have important roles in this process. *In vitro*, IGF-1 and insulin can stimulate theca cells androgen synthesis [Barbieri *et al.*, 1986]. IGF-II and IGF-binding proteins (IGFBPs) are thought to play a modulatory role in androgen production by thecal cells. FSH has been shown to

stimulate IGF-I production by granulosa cells. Activins and inhibins also regulate theca androgen production [Hillier *et al.*, 1991]. Inhibin can inhibit FSH production through negative feedback and has been speculated to be important in pre-ovulatory follicle development [Adashi *et al.*, 1991]. Activin predominates in immature follicles, promoting FSH-induced mitosis and steroidogenic differentiation.

In mono-ovulatory mammals such as humans, only 1 follicle is selected to achieve dominant follicle status, with the other recruited follicles undergoing atresia. Atresia is thought to occur due to a number of metabolic and /or genetic defects in the follicle [reviewed by Moley and Schreiber, 1995]. Moreover, anti-apoptotic and pro-apoptotic proteins have been shown to affect oocyte and primordial follicle numbers in the mouse ovary. Bcl2 knockout-mice studies have implicated these anti-apoptotic proteins in oocyte and primordial follicle survival. Bax knock-out mice studies have revealed its importance in ovarian somatic-cell death [reviewed by Tilly, 1995]. However, the precise cellular mechanisms underlying atresia still remain unclear.

1.1.2.2 Ovulation

Following dominant follicle selection, the increase in oestradiol production (through the acquisition of LH receptors by the granulosa cells of the dominant follicle) leads to a decline in FSH secretion. At this stage, further follicle growth becomes LH dependent. The further increase in oestradiol production causes a positive feed-forward loop on the release of FSH and LH from the anterior pituitary gland. This results in an increased pulsatile frequency of LH and causes the mid-cycle

gonadotrophin surge. The resulting increase in release of LH is thought to stimulate both physical and biochemical changes in the follicle through the induction of gene expression [Richards *et al.*, 1994; Tsafiriri and Reich 1999, Espey and Richards 2002]. The biochemical changes include the up-regulation of the progesterone receptor gene, and the induction of prostaglandin H synthase 2 (PGHS2) gene expression. These changes in turn induce the expression of matrix metalloproteinases (MMPs), synthesis of growth factors, and the recruitment of inflammatory cells [Petrovska *et al.*, 1992; Buksovsky *et al.*, 1995; Brannstrom and Friden 1997], to mediate this inflammatory event of ovulation [Espey, 1980]. These changes result in follicular rupture, and expulsion of the oocyte from the follicle. The expression of glucocorticoid-metabolising 11β hydroxysteroid dehydrogenase enzymes are also regulated at the time of ovulation; this will be discussed further in section 1.8 [Tetsuka *et al.*, 1997; 1999a]. Several structural changes are associated with ovulation, these include the degradation of the extracellular matrix at the follicular apex, and vascularisation of the follicle.

1.1.2.3 Follicular Fluid

The antrum of the ovarian follicle, filled with an aqueous substance termed follicular fluid (FF), is formed during early folliculogenesis. FF is comprised of many lipids, proteins, growth factors, respiratory substrates and various as yet undetermined factors (Sutton *et al.*, 2003). The components of FF are derived from serum exudate modified by secretions from both the granulosa cells and the oocyte (Sutton *et al.*, 2003). The basal lamina between the vascularised theca interna and the granulosa cells also acts a filtering system of serum both on molecular weight (e.g. slowing

entry of LDL into the follicular antrum) and on the basis of molecular charge (Rodgers *et al.*, 2003). Coupled to this, channels exist between the granulosa cells which can potentially let in proteins with a molecular weight of up to 500 kDa (Rodgers *et al.*, 2003).

As the mid-cycle surge of LH occurs the follicle undergoes a number of structural and functional changes, the result of which lead to a change in follicular fluid composition. An increase in the angiogenic factor VEGF has been detected in FF following the pre-ovulatory surge of LH (Anasti *et al.*, 1998). This promotes vascularisation coupled with an increase in vascular permeability of the follicle, resulting in an increase of plasma influx into the FF. As a consequence, as ovulation approaches, the representation of plasma proteins in FF increases. On the other hand, in the final stages of folliculogenesis, LH and FSH exert the greatest action on the local ovarian synthesis of both steroid and peptide hormones, such that these compounds (e.g. oestradiol, progesterone and inhibin) tend to be much more concentrated in the FF of mature pre-ovulatory follicles than in plasma.

In the preovulatory follicle, LH also increases the production of hyaluronan by the cumulus cells of the cumulus-oocyte complex. This loosens the hyaluronic acid matrix between neighbouring cumulus cells and so is essential for the normal expansion of the cumulus-oocyte complex (Rodgers *et al.*, 2003; Jessen and Odum 2003). Emerging literature has shown that the increase in basal lamina permeability is essential for the influx into FF of plasma proteins that are essential for hyaluronan formation. One such plasma protein, inter α -trypsin inhibitor, is thought to couple to hyaluronan and stabilise the extracellular matrix of the cumulus-oocyte complex

(Jessen and Odum, 2003). Thus revealing the changes in follicular dynamics and FF composition that LH induces in the preovulatory follicle.

1.1.2.4 The Luteal Phase

Following ovulation, the remaining follicular cells undergo extensive remodelling to form the corpus luteum (CL). The process of CL formation involves irreversible follicular remodelling with the theca and granulosa cells differentiating into a luteal phenotype. These cells become highly vascularised through proliferation of capillaries from surrounding stromal cells, which penetrate the basal lamina [Duncan 2000]. Vascularisation is controlled by a number of angiogenic factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [Gospodarowicz 1985; Reynolds *et al.*, 1998]. These factors also induce endothelial cell migration. Many proteases are activated, such as MMPs, which alter the shape of the forming CL.

During luteinisation, the expression of a number of genes and proteins associated with steroidogenesis occurs [Richards *et al.*, 1998]. Examples of such genes include components of the cholesterol uptake and transport system such as steroidogenic acute regulatory (StAR) protein and sterol carrier protein 2 (SCP-2). As luteinisation occurs, the primate CL produces progesterone and oestradiol under the influence of LH [Channing 1980]. Progesterone and oestradiol act in concert to inhibit gonadotrophin secretion from the pituitary, and therefore control the onset of the next ovarian cycle. In response to progesterone production by the CL, the endometrium secretes nutrient factors to support an embryo should fertilisation occur

[Spencer and Bazer 2002]. In the event of fertilisation, the CL persists to maintain circulating progesterone concentrations until the placenta is established. Progesterone also acts to enhance the negative feedback effects of oestradiol and inhibin on FSH and LH release and to block the positive feedback effects of oestradiol [Schaison and Couzinet 1991; Leung and Peng 1996].

In the absence of fertilisation the CL regresses. The exact mechanisms controlling the lifespan of the CL and luteal regression remain unclear. A number of structural and functional changes are involved in CL regression. These include membrane breakdown as indicated by decreased membrane fluidity [Carlson *et al.*, 1982; Greenhalgh 1990; Riley and Behrman 1991] coupled with an increase in phospholipase A₂ (PLA₂) activity [Sawada and Carlson 1991]. There are known luteolytic and luteotrophic factors such as prostaglandin (PG)F_{2α} and PGE₂ that can shorten or prolong the lifespan of the CL respectively. The synthesis of such eicosanoids are enhanced by the increase in PLA₂ activity.

1.2 Ovarian Steroidogenesis

1.2.1 Cholesterol uptake and transport

Cholesterol is the precursor of steroid biosynthesis. Cholesterol can be obtained from 4 sources: as free cholesterol or cholesteryl esters from plasma lipoproteins, as intracellular cholesteryl esters stored in lipid droplets, as free cholesterol from the plasma membrane, or by *de novo* synthesis.

Steroidogenic tissues, including the ovary, acquire cholesterol substrate primarily through the uptake of plasma lipoproteins containing cholesteryl esters and free cholesterol. Three types of lipoproteins have been identified: high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) [Grummer and Carroll, 1988]. In humans, the primary source of cholesterol is from LDL [Gwynne and Strauss, 1982], providing the majority of cholesterol for steroidogenesis.

Receptor-mediated endocytosis is the predominant mechanism through which LDL-derived cholesterol can enter cells [Rhainds and Brissette, 1999]. On internalisation into the cell, the LDL receptor is either recycled to the plasma membrane or degraded. The resulting LDL particle is taken up by lysosomes, leading to degradation of LDL and the liberation of free cholesterol. On entering the cell, cholesterol can either be stored in its esterified form, or liberated from stored cholesteryl esters depending on the requirement of the cell. There are 2 enzymes that control the equilibrium of free and esterified cholesterol in the cell: acyl coenzyme A cholesterol acyltransferase (ACAT) and cholesterol esterase. ACAT favours the storage of cholesterol in its esterised form converting free cholesterol to cholesteryl esters, and cholesterol esterase liberates free cholesterol from cholesteryl esters [Flint and Armstrong, 1973].

Once in the cell, in order for the first step of steroidogenesis to occur, cholesterol needs to be transported from the cytoplasm through the outer mitochondrial membrane into the inner mitochondrial membrane, where cholesterol is converted to pregnenolone by cytochrome P450_{csc} (CYP11A). To transport cholesterol from the

cytosol to the mitochondria, methods of facilitated transport are required due to the hydrophobic properties of cholesterol. The cytoskeleton and sterol carrier protein 2 (SCP₂) are both thought to play a role in this process. Various constituent parts of the cytoskeleton (e.g. tubulin) have been linked to increased steroidogenesis. It has been hypothesised that changes in cell shape can increase steroidogenesis through shortening the distance that cholesterol needs to travel to the mitochondria for conversion to pregnenolone. Studies in Y1 adrenocortical cells have shown that increased steroidogenesis is associated with changes in cell morphology, namely rounding of the cells [Betz and Hall, 1987]. This evidence suggests the possible role of cytoskeletal re-organisation in cholesterol transport.

SCP₂ is an aqueous carrier protein that has been speculated to play a role in cholesterol transport. Studies have revealed that anti-SCP₂ antibodies can decrease steroid synthesis [Chanderbhan *et al.*, 1986]. SCP₂ transcripts have been localised to the ovary, and trophic hormones that increase steroidogenesis have been shown to increase SCP₂ gene expression [Rennert *et al.*, 1991].

There are two main mechanisms of cholesterol transport from the outer mitochondrial to the inner mitochondrial membrane. The peripheral benzodiazepine receptor (PBR) has been shown to be one mechanism that facilitates cholesterol transport. PBR expression is abundant in steroidogenic tissue, and in the ovary, the expression of PBR has been shown to vary across the ovarian cycle, with maximal expression observed following ovulation [Gavish, 1995]. The ligand for PBR is diazepam-binding inhibitor (DBI), the binding of which to PBR stimulates mitochondrial cholesterol transport [Bar-Ami *et al.*, 1991]. Binding of DBI to the

PBR is essential for cholesterol transport, such that the depletion of DBI has been shown to inhibit constitutive and hormone stimulated-steroidogenesis in Leydig cells [Boujrad *et al.*, 1993].

StAR protein has also been implicated in mitochondrial cholesterol transport. StAR was first cloned by Clark *et al.* [1994] and numerous studies have since investigated its role in cholesterol transport. In humans, a mutation in the StAR gene results in a potentially lethal phenotype termed congenital lipoid adrenal hyperplasia [Lin *et al.*, 1995]. This results in a reduction of gonadal and adrenal steroids. StAR knock-out mice studies also reveal a similar phenotype [Caron *et al.*, 1997]. An explanation for the mechanisms by which StAR mediates cholesterol transport was provided by proposing that StAR targets cholesterol to the PBR, which then mediates cholesterol transport to the inner mitochondrial membrane [West *et al.*, 2001].

1.2.2 Ovarian Steroidogenesis

The first step of steroidogenesis is the conversion of cholesterol to pregnenolone by P450_{csc} (Figure 1.1). This reaction takes place in the inner mitochondrial membrane and involves 3 reactions, the first 2 steps leading to the hydroxylation of C22 and C20 of cholesterol, and the third resulting in the cleavage of the C20-C22 bond to yield the C21 product pregnenolone, and the C6 molecule, isocaproic acid [Burstein and Gut, 1971]. Each reaction utilises nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor (Figure 1.1).

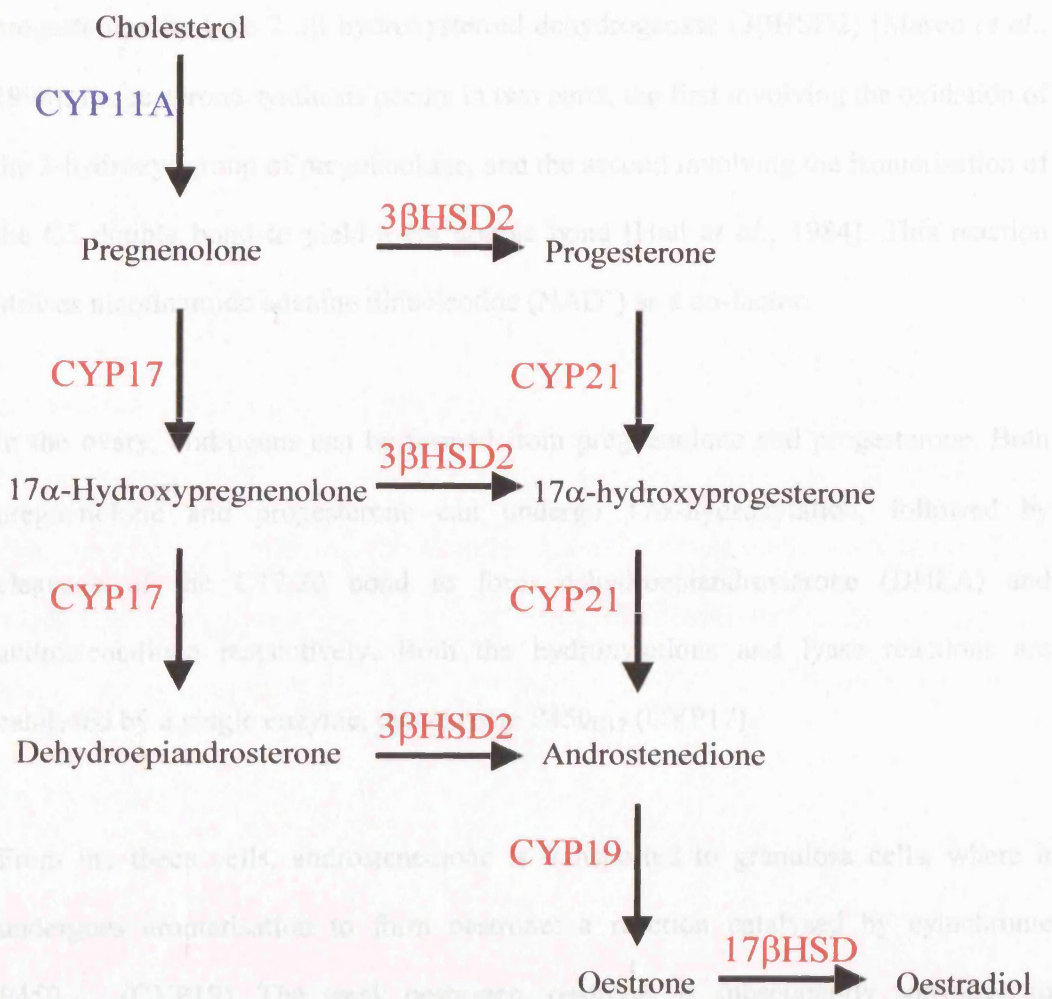


Figure 1.1: Principle pathways of ovarian steroidogenesis. Blue script- mitochondrial reactions, red script- ER reactions.

Once pregnenolone is formed, it leaves the inner mitochondrial membrane and moves to the smooth endoplasmic reticulum (ER) where it is converted to progesterone by type 2 3β hydroxysteroid dehydrogenase (3β HSD2) [Mason *et al.*, 1998]. Progesterone synthesis occurs in two parts, the first involving the oxidation of the 3-hydroxyl group of pregnenolone, and the second involving the isomerisation of the C5 double bond to yield a C4 double bond [Hall *et al.*, 1984]. This reaction utilises nicotinamide adenine dinucleotide (NAD^+) as a co-factor.

In the ovary, androgens can be formed from pregnenolone and progesterone. Both pregnenolone and progesterone can undergo 17α -hydroxylation, followed by cleavage of the C17,20 bond to form dehydroepiandrosterone (DHEA) and androstenedione respectively. Both the hydroxylations and lyase reactions are catalysed by a single enzyme, cytochrome P450_{C17} (CYP17).

From the theca cells, androstenedione is transported to granulosa cells, where it undergoes aromatisation to form oestrone: a reaction catalysed by cytochrome P450_{arom} (CYP19). The weak oestrogen, oestrone, is subsequently converted to oestradiol by 17β HSD1. The aromatisation of androgens to oestrogens in granulosa cells is controlled by FSH. The process of thecal cell androgen production, and the subsequent conversion to oestrogens by granulosa cells is termed the 2-cell, 2-gonadotrophin hypothesis [Hillier *et al.*, 1984; Hillier *et al.*, 1994]. As noted in section 1.1.2, many intra-ovarian and paracrine factors have been implicated in the control of this process including TGF- β , and IGFs [Hillier *et al.*, 1994].

Although the ovary does not express the CYP21 enzyme necessary for *de novo* synthesis of cortisol, it can generate cortisol from cortisone. The enzyme required for this reaction, an isoform of 11 β HSD is known to be expressed in human [Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Smith *et al.*, 1997; Ricketts *et al.*, 1998; Smith *et al.*, 2000], bovine [Tetsuka *et al.*, 2003] and rat [Tetsuka *et al.*, 1999a] ovaries and appear to be differentially regulated across the ovarian cycle (see section 1.8).

1.3 Eicosanoids and Ovarian function

Eicosanoids are hydrophobic inflammatory molecules derived from arachidonic acid (AA) and control ovarian function in a paracrine fashion. Eicosanoids have established roles in ovulation and in regulating the lifespan of CL. However, before exploring these roles, the biosynthetic pathways of eicosanoid synthesis will be outlined.

1.3.1 Prostaglandin Biosynthesis

All prostaglandins are comprised of a prostanoid fatty acid skeleton. The position on the fatty acid skeleton at which the oxygen containing moieties occur confer the biological activity of each prostaglandin, with positions 9, 11, and 15 being the most significant positions in terms of biological function. Three categories of prostaglandins have been identified termed 1-, 2-, and 3- series prostaglandins. Each series differs in their degree of saturation and is formed from different polyunsaturated fatty acid precursors.

The most biologically important precursor for prostaglandins is AA which is stored in cell membranes, esterified at the C2 position of membrane phospholipids. Three alternative pathways control the release of AA from the lipid membrane: the phospholipase A₂ (PLA₂) pathway, the phospholipase C (PLC) pathway, and the phospholipase D (PLD) pathway. Each pathway involves hydrolysis of the phospholipid head-group to liberate AA.

The first step of AA conversion to prostaglandins is catalysed by isoforms of the enzyme prostaglandin H synthase (PGHS, formerly named cyclo-oxygenase). This enzyme is a haem-containing protein that has 2 catalytic activities; a cyclo-oxygenase activity, and an endoperoxide synthase activity. The cyclo-oxygenase activity catalyses the addition of 2 oxygen atoms to AA to form PGG₂. The endoperoxide synthase activity converts the hydroperoxy moiety of PGG₂ to a hydroxyl group, forming PGH₂. Hence, PGHS represents the first irreversible step in the prostanoid biosynthetic pathway [reviewed by Smith *et al.*, 1996].

To date, there are 3 cloned isoforms of PGHS. PGHS3 is thought to be a splice variant of PGHS1 and has been isolated from canine and human cerebral cortex, although little is known about its function [Chandrasekharan *et al.*, 2002]. Although functionally different, PGHS1 and PGHS2 have very similar structures. Both form homodimers, and are haem-containing, glycosylated, integral membrane proteins, sharing the same catalytic mechanism. Both PGHS1 and PGHS2 have the same affinity for AA ($K_m \sim 5 \mu\text{M}$) [Barnett *et al.*, 1994] with subtle differences in substrate fatty acid specificities and peroxide requirements [Laneuville *et al.*, 1995]. Both PGHS1 and PGHS2 are anchored to the luminal face of the ER, luminal surfaces of

the nuclear envelope and contiguous outer membrane of the nuclear membrane. These enzymes attach to the luminal leaflet of these specific membranes through the hydrophobic surfaces of amphipathic helices within the protein structures of the enzymes.

PGHS1 is a constitutive enzyme. It is ubiquitously expressed, and is typically expressed at constant levels throughout the cell cycle. The function of PGHS1 appears to be in maintaining renal functions and platelet function [reviewed by Smith *et al.*, 1996]. In contrast PGHS2 is an inducible enzyme that is virtually undetectable in most tissues. However, rapid activation of PGHS2 expression can be induced within 2-6 hours of hormone, growth factor or cytokine stimulation. Following induction, PGHS2 mRNA expression has been detected in many tissues including ovarian follicles [Sirois *et al.*, 1992]. Moreover, anti-inflammatory agents, such as cortisol, have been shown to decrease PGHS2 mRNA and protein expression via transcriptional and post-transcriptional mechanisms [reviewed by Smith *et al.*, 1996].

The functional roles of PGHS2 are thought to be two fold. It is thought that the enzyme co-localises with PGHS1 on the luminal face of the ER to augment the function of PGHS1. The localisation of PGHS2 to the luminal face of the nuclear envelope is speculated to operate as a unique nuclear prostanoid biosynthetic pathway that forms prostaglandins, which can act via the nuclear membrane, or nucleoplasmic targets in association with cell differentiation and replication. Indeed, prostaglandins are ligands for the members of the peroxisome proliferator activated receptor (PPAR) family of nuclear receptors [Helliwell *et al.*, 2004].

Once PGH₂ has been formed, it undergoes various structural changes to form PGD₂, PGE₂, PGF_{2α}, PGI₂ or thromboxane A₂ (TXA₂). The resulting prostanoids exert their cellular actions through paracrine mechanisms. Each prostaglandin is secreted and can bind to G-protein coupled receptor (GPCR) on neighbouring cells, and activate various second messenger signalling cascades including cAMP-protein kinase A (PKA) (activated by PGE₂) and calcium-calcium dependent protein kinase (PKC) (stimulated by PGF_{2α}) [reviewed by Narumiya *et al.*, 1999]. Prostaglandins are also thought to signal through intracrine mechanisms, specifically through binding to orphan nuclear receptors such as PPARs [reviewed by Helliwell *et al.*, 2004]. A role for the nuclear generation of prostaglandins has also been speculated. Stimulation of PGHS2 with IL-1β was shown to re-localise PGHS2 from the nucleus to the nuclear envelope and the cytoplasm, with no effect on PGHS1 localisation [Parfenova *et al.*, 2001], highlighting novel mechanisms through which prostaglandins may exert their actions.

1.3.2 Lipoxygenase

The lipoxygenase pathway of AA metabolism to leukotrienes involves the oxidation of AA to form hydroperoxy-eicosatetraenoic acids (HPETEs) and hydroxyeicosatetraenoic acids (HETEs). HETEs and HPETEs can be further reduced to leukotrienes according to the requirements of the cell [Ueda *et al* 1986; Samuelsson, 1987].

1.3.3 Epoxygenase

The actions of epoxygenase metabolises AA to generate dihydroxyacids and epoxyeicosatrienoic acids [Zeldin, 2001]. The involvement of these metabolites in phospholipase A₂ intracellular signalling are yet to be elucidated [Van Voorhis *et al* 1993].

1.3.4 Role of Eicosanoids in Ovulation

The administration of non-selective PGHS inhibitors to human and non-human primates has been shown to inhibit ovulation [Wallach *et al.*, 1975a; Killick and Elstein 1987]. Moreover, in non-human primates, ovulation could be restored by simultaneous administration of PGF_{2α} [Wallach *et al.*, 1975b]. Similar studies in rodents using non-selective PGHS inhibitors either administered systemically [Tsafriri *et al.*, 1972; Mikuni *et al.*, 1998] or into perfused whole ovaries [Hamada 1978; Sogn *et al.*, 1987; Mikuni *et al.*, 1998] inhibited ovulation. Subsequent co-administration of PGE₂ or PGF_{2α} was shown to restore the ovulatory process. Taken together, these data have supported the hypothesis that ovulation is an inflammatory event, mediated, in part, by prostaglandins [Espey 1980].

Further studies into the role of the PGHS isoform in ovulation have shown that the administration of preferential PGHS2 inhibitors disturbs ovulation in women by preventing (or delaying) follicular rupture, despite normal profiles of oestradiol and progesterone [Smith *et al.*, 1996; Mendonca *et al.*, 2000; Pall *et al.*, 2001]. Knock-out mice studies have shown that PGHS1 null mice exhibit normal ovulation and fertilisation [Langenbach *et al.*, 1995], whereas, PGHS2 null mice show a decrease

in the number of ovulation sites [Lim et al., 1997]. Moreover, administration of PGE₂ to PGHS2 knockout mice restores the normal ovulatory pattern.

Studies in the rhesus macaque found that 12 hours post administration of an ovulatory dose of human chorionic gonadotrophin (hCG), PGHS2 mRNA expression was induced in follicular granulosa cells, and remained elevated through to 36 hours post hCG administration [Duffy and Stouffer, 2001]. PGHS1 mRNA did not change at 0, 12, and 36 hours post hCG. The concentrations of PGE₂ and PGF_{2α} in follicular fluid (FF) did not change over the first 24 hours, but were 100-fold higher by 36 hours post hCG administration [Duffy and Stouffer, 2001]. These data agree with the proposal by Sirois and Dore [1997] that the increase in FF prostaglandins is the determining step controlling ovulation in mammals.

Use of immunohistochemistry in the rhesus macaque follicle after administration of an ovulatory dose of hCG, localised PGHS2 expression to the theca and granulosa cells. This was also confirmed in naturally cycling animals [Duffy and Stouffer, 2001]. In contrast PGHS1 expression was only detected in ovarian surface epithelial (OSE) cells [Duffy and Stouffer, 2001] where PGHS1 is speculated to play a role in apoptosis which is essential for follicular rupture. This suggestion has been supported through administration of indomethacin, an inhibitor of PGHS1 and PGHS2, which was shown to inhibit apoptosis in OSE cells [Murdoch, 1996].

Knockout mice studies of PGE₂ (EP) receptors and the PGF_{2α} (FP) receptor have yielded different results. EP2 receptor or PGHS2 knock-out mice exhibit defects in ovulation rate and cumulus cell expansion, suggesting alternative roles of

prostaglandins in the control of ovarian function [Tilley *et al.*, 1999; Hizaki *et al.*, 1999]. However, other studies have shown that EP or FP receptor knockout mice show no defect in ovulation [Kobayashi and Narumiya, 2002], highlighting the need for further study to elucidate the role for prostaglandins in ovulation. Moreover, PGE₂ and PGF_{2α} have been shown to play critical roles in oocyte maturation [Watanabe *et al.*, 1994], suggesting other mechanisms of ovarian function that prostaglandins are important not only in ovulation, but also in folliculogenesis.

Products of the lipoxygenase pathway have also been implicated in the ovulatory process. The presence of leukotriene B₄ (LTB₄) has been detected in human FF, and lipoxygenase activity has been detected in human granulosa-lutein cells from women undergoing *in vitro* fertilisation and embryo transfer (IVF-ET) [Feldman *et al.*, 1986; Priddy *et al.*, 1989]. Reich *et al.* [1983] demonstrated that various inhibitors of lipoxygenase could block ovulation in the rat. Further studies revealed the presence of 12-HETE [Espey *et al.*, 1991] and 15-HETE [Tanaka *et al.*, 1989; Espey *et al.*, 1991; Tanaka *et al.*, 1991] in the rat ovary following hCG administration. Other studies have detected increases in LTB₄ following administration of an ovulatory dose hCG to perfused rabbit ovaries [Yoshimura *et al.*, 1991]. Administration of a preferential lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), to perfused rabbit ovaries following an ovulatory dose of hCG reduced ovulatory efficiency. However, co-administration of LTB₄ reversed the inhibitory effects of NDGA on ovulation [Yoshimura *et al.*, 1991; Mikuni *et al.*, 1998]. Inhibitor studies of the lipoxygenase pathway using indomethacin, NDGA and MK-886 have all been shown to decrease the expression of MMP-1 and MMP-2 in rat ovarian extracts

following hCG administration. This indicates a role for lipoxygenase products in follicular collagenase stimulation [reviewed by Tsafiriri 1995].

Speculated roles for lipoxygenase products in ovulation are similar to those for prostaglandins. Lipoxygenase metabolites of AA are speculated to affect vascular permeability, vasodilation and collagenolysis.

1.3.3 Role of eicosanoids in CL function

Luteal cells and tissues have the capacity to synthesise prostaglandins in all mammalian species investigated including cows [Milvae and Hansel 1983; Pate 1988], pigs [Watson and Patek 1979], rats [Tamura *et al.*, 1991; Olofsson *et al.*, 1992], non-human primates [Houmard and Ottobre 1989] and women [Challis *et al.*, 1976]. Coupled to this, PLA₂ activity has been shown to increase throughout luteolysis in rats [Wu and Carlson 1990] and abrogate steroidogenesis in luteal cells [Greenhalgh 1990]. Radioimmunoassays of tissue from the rat CL throughout pseudopregnancy and pregnancy have detected the presence of PGE₂, PGF_{2α}, and PGI₂ [Olofsson *et al.*, 1990]. In other species, similar findings have been obtained [Miller *et al.*, 1983; Vijayakumar and Walters 1987]. The FP receptor has been localised to the CL in all species tested [Reviewed by Anderson *et al.*, 2001]. Functional EP1 and EP2 receptors have also been identified in human granulosa-lutein cells [Harris *et al.*, 2001].

There are 2 ways in which prostaglandins are thought to control steroidogenesis and the functional lifespan of the CL. PGE₂ is thought to act as a luteotrophin,

functioning to stimulate progesterone synthesis. $\text{PGF}_{2\alpha}$ may act in a luteolytic manner, to inhibit progesterone synthesis. However, the role of $\text{PGF}_{2\alpha}$ in luteolysis in primates remains unclear.

Indomethacin treatment for seven days during the early and mid-luteal phase has been shown to decrease both basal and GnRH-stimulated progesterone concentrations, indicating a luteotrophic role for prostaglandins and lipoxygenase products within the early and mid-luteal phase [Fulghesu *et al.*, 1993].

The luteotrophic role of PGE_2 has been demonstrated in both human [Dennefors *et al.*, 1982; Hahlin *et al.*, 1988] and bovine [Shelton *et al.*, 1990; Sharif *et al.*, 1998] CL. Moreover, the luteotrophic actions of PGE_2 have been demonstrated in luteinised granulosa cells [McNatty *et al.*, 1975], and luteal cells of the rhesus macaque [Stouffer *et al.*, 1979], rat [Thomas *et al.*, 1978] and hamster [Yang *et al.*, 1998]. In the human [Dennefors *et al.*, 1982; Hahlin *et al.*, 1988] and non-human primate CL [Molskness *et al.*, 1987], progesterone production was found to increase in response to PGE_2 apparently acting via the stimulation of adenylyl cyclase, and increased generation of cAMP.

In the rat CL, $\text{PGF}_{2\alpha}$ can inhibit LH-stimulated adenylyl cyclase activity, partly due to the impairment of the G_s -protein [Lahav *et al.*, 1976; Norjavaara and Rosberg 1986]. This inhibition was shown to increase with luteal age [Khan *et al.*, 1979]. Moreover, in human granulosa-lutein cells, $\text{PGF}_{2\alpha}$ can inhibit LH-stimulated steroidogenesis both prior and subsequent to the elevation of cAMP [Michael *et al.*, 1993]. Other studies have also highlighted temporal differences in signalling

pathways coupled to the FP receptor in luteal cells, one of which is the calcium-dependent PKC signalling pathway [Steele and Leung, 1993].

1.4 Role of LH in Ovarian function

LH is a glycoprotein hormone produced alongside FSH by the gonadotrophs of the anterior pituitary. LH is a heterodimer consisting of an α -subunit that is non-covalently bonded to a β -subunit. The pattern of glycosylation confers the functional specificity on LH [reviewed by Ascoli *et al.*, 2002].

The LH receptor (LHR) is a single polypeptide with an overall structure and topology of members of the rhodopsin/ β -adrenergic superfamily of GPCRs [Segaloff *et al.*, 1990]. The receptor comprises a large N terminal extracellular region consisting of approximately 340 residues, a 7 transmembrane domain, and a C terminal intracellular region.

The LHR couples to the adenylyl cyclase signalling cascade [Richards *et al.*, 1998]. Prior to ovulation, LH activates a cAMP-PKA signalling cascade to induce the expression of genes required for cell proliferation and differentiation. As the mid-cycle LH surge occurs, triggering ovulation, LH dramatically increases cAMP-PKA signalling. LH can also increase inositol lipid-hydrolysis, which results in elevation of the intracellular calcium concentration and activation of PKC activation. This in turn alters the pattern of gene expression to orchestrate the final stages of follicular development, causing the oocyte to undergo meiotic maturation, and rupture of the follicular wall [Robker and Richards 1998; Espey and Richards 2002]. Genes that

are up-regulated by LH include interleukin (IL)-1 β , the IL-1 β receptor (IL-1 β R) [Adashi, 1997; 1998] and PGHS2 [Morris and Richards 1995]. Little is known about the transcription factors and regulators that mediate these responses, however, C/EBP is speculated to play a role [Sterneck *et al.*, 1997].

The expression of the LHR has been localised to various tissue types including the ovary, specifically in the thecal cells, interstitial cells, differentiated granulosa cells, and luteal cells [reviewed by Ascoli *et al.*, 2002]. *In situ* hybridisation studies have localised LHR mRNA to the thecal and interstitial cells in immature pre-antral follicles. In mature antral follicles, LHR mRNA is localised to both thecal and granulosa cells [Camp *et al.*, 1991]. Expression of LHR mRNA in the granulosa is increased by FSH administration *in vitro* and FSH has been shown to directly regulate LHR gene transcription [Shi and Segaloff 1995].

LH is an integral factor in the control of the ovarian cycle. In the follicular phase, LH controls thecal cell androgen production with aromatisation of androstenedione to form oestrogens in the neighbouring granulosa cells [Hillier *et al.*, 1994]. The increase of LH pulsatile frequency during the peri-ovulatory phase induces essential genes for ovulation [Espey and Richard, 2002]. During the luteal phase, LH is a key luteotrophic factor, stimulating progesterone production and supporting CL function [Kohen *et al.*, 2003]. LH plays a key role in the regulation of prostaglandin synthesis through regulating the expression of PGHS2 (reviewed in section 1.3).

1.5 Role of Interleukin-1 in Ovarian function

Preliminary studies into the role of IL-1 in ovarian function detected IL-1 activity in human FF [Khan *et al.*, 1988]. Subsequent studies localised both IL-1 α and IL-1 β mRNA to the cumulus cells of women undergoing IVF-ET [Barak *et al.*, 1992; De Los Santos *et al.*, 1998]. Recent *in vitro* studies have confirmed that human granulosa cells secrete IL-1 β [Carlberg *et al.*, 2000]. Moreover, studies have correlated intrafollicular levels of IL-1 with oocyte quality in women undergoing IVF-ET [Karagouni *et al.*, 1998, Mendoza *et al.*, 1999].

Two receptor subtypes for the IL-1 system have been cloned [reviewed by Gerard *et al.*, 2004]. The IL-1R couples to p38 mitogen activated protein kinase (MAPK) and Jun N-terminal kinase (JNK) signalling pathways, resulting in the activation of nuclear factor- κ B (NF- κ B) [O'Neill, 2000]. The type I IL-1R has been localised to human granulosa, cumulus, theca cells [Hurwitz *et al.*, 1992] and the human embryo [De Los Santos *et al.*, 1998]. To date, studies to characterise the expression of the type II IL-1R in the ovary have been limited. However, the type II IL-1R has been localised to equine cumulus cells and oocytes both before and after *in vitro* maturation. In rat immature ovaries the type II receptor was not detected, although it was detected in cultured ovarian cells [reviewed by Gerard *et al.*, 2004].

The naturally occurring IL-1 receptor antagonist (RA), has been shown to regulate the bioavailability of IL-1 α and IL-1 β to their receptors. IL-1RA is known to be expressed in the human ovary [Hurwitz *et al.*, 1992]. Subsequent studies have localised IL-1RA to human granulosa cells, which were devoid of any

immunological cells suggesting *de novo* synthesis of IL-1RA by granulosa cells [Kol *et al.*, 1999]. The IL-1RA has also been localised to human cumulus cells, with its quantity changing throughout follicular maturation [Tsafiriri, 1995].

The various components of the IL-1 system are thought to regulate ovarian function. There are hypothesised roles for IL-1 in ovulation, oocyte maturation, cellular metabolism, and ovarian steroidogenesis. IL-1 β has been shown to increase progesterone production in human granulosa cells. In non-ovarian tissue, IL-1 promotes many ovulation-associated physiological and biochemical changes including collagen activation, increased vascular permeability and prostaglandin production [Gerard *et al.*, 2004].

In studies across various species, IL-1 has been shown to stimulate PGE₂ and PGF_{2 α} production. Studies by Narko *et al.* [1997] have revealed that IL-1 β can induce PGHS2 mRNA expression in human granulosa-lutein cells. Moreover, subsequent studies revealed that IL-1 β can induce the expression of mRNA encoding FP, EP2 and EP4 receptors in hGL cells [Narko *et al.*, 2001]. In human FF the concentration of IL-1 β has been correlated with the concentration of PGE₂ and PGF_{2 α} [Watanabe *et al.*, 1994] providing evidence of a regulatory link between IL-1 β and prostaglandin synthesis via PGHS2. Other studies have also shown that IL-1 β can increase PLA₂ activity [Kol *et al.*, 1997], providing AA, the substrate of prostaglandin synthesis.

Studies into the effect of IL-1 β on the expression of PGHS2 in human granulosa-lutein cells harvested during different phases of the ovarian cycle has shown that IL-1 β can induce the expression of PGHS2 mRNA and decrease its degradation [Narko *et al.*, 1997]. These data highlight the importance of IL-1 β in the induction of PGHS2 for prostaglandin synthesis in the ovary.

1.6 Cortisol Metabolism

1.6.1 Synthesis of glucocorticoids

Glucocorticoids are synthesised in the adrenal cortex, specifically in the zona fasciculata in response to adrenocorticotrophic hormone (ACTH) stimulation. As for all steroids, the substrate for glucocorticoid synthesis is cholesterol (Figure 1.2). As outlined in section 1.2.2, cholesterol is converted to pregnenolone by CYP11A (P450_{csc}), and pregnenolone is oxidised to progesterone by 3 β HSD. Once formed, progesterone can be converted to cortisol through a 3 step process. Firstly, progesterone is converted to 17 α -hydroxyprogesterone by CYP17. This 17 α -hydroxyprogesterone is then converted to 11-deoxycortisol by CYP 21 (21-hydroxylase). The final step in the reaction is catalysed by 11 β -hydroxylase (CYP11B1) converting 11-deoxycortisol to cortisol (Figure 1.2) [reviewed in Brook and Marshall 2001].

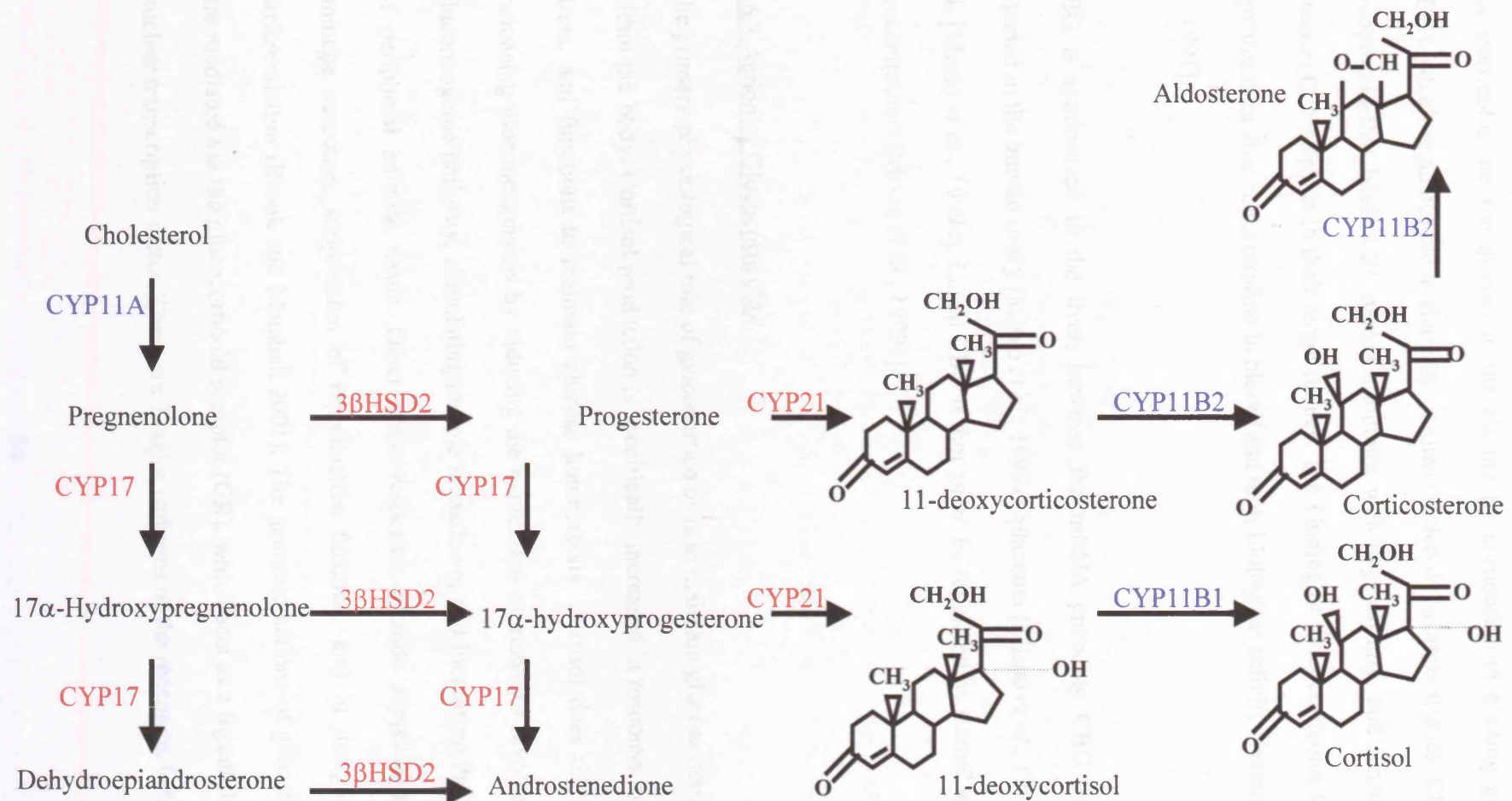


Figure 1.2: Principle pathways of glucocorticoid biosynthesis. Blue=mitochondrial pathways, red=ER reactions, HSD=hydroxysteroid dehydrogenase, CYP= cytochrome P450

1.6.2 Circulation of glucocorticoids

Glucocorticoids are transported in the plasma by corticosteroid binding globulin (CBG), which regulates the availability of glucocorticoids to target tissues. CBG is a glycoprotein that binds C21 steroid hormones with high affinity, and functions to transport C21 steroids to their target tissues. The binding of glucocorticoids to CBG regulates their free concentration in blood, and their biological activity [Hammond *et al.*, 1991].

CBG is synthesised in the liver, however the mRNA encoding CBG has been detected in the human ovary [Misao *et al.*, 1999a], placenta [Misao *et al.*, 1999b] and CL [Misao *et al.*, 1999c]. Luteal CBG is thought to be regulated by steroid hormone concentrations [Misao *et al.*, 1999c].

1.6.3 Action of Glucocorticoids

The primary physiological role of glucocorticoids is to maintain glucose homeostasis within the body. Cortisol production is specifically increased in response to chronic stress, and functions to maintain glucose homeostasis. Cortisol does this through increasing gluconeogenesis by inducing the expression of enzymes involved in the gluconeogenic pathway, stimulating muscle breakdown, and increasing the turnover of peripheral adipose tissue. Other stress responses include suppression of the immune response, suppression of reproductive function, and in instigating cell differentiation [Brook and Marshall 2001]. The genomic actions of glucocorticoids are mediated via the glucocorticoid receptor (GR), which acts as a ligand-dependent nuclear transcription factor. There are 2 major isoforms of the receptors, GR α , which

is transcriptionally active, and GR β , which acts as a dominant negative regulator of transcription [Charmandari *et al.*, 2004]. Recent publications have suggested that the ligand- and tissue- specific effects of the GR are mediated through translational variants of the receptor isoforms [Yudt and Cidlowski 2002; Lu and Cidlowski 2004].

1.6.4 Glucocorticoid-metabolising enzymes

There are 2 important glucocorticoid-metabolising systems that regulate the activity of cortisol, namely 11 β -hydroxysteroid dehydrogenase (11 β HSD), and the 5 α /5 β -reductase pathways. In glucocorticoid target tissues, the active glucocorticoid cortisol (corticosterone in mice and rats) can be inter-converted to its inactive metabolite cortisone (11-dehydrocorticosterone in mice and rats), by isoforms of 11 β HSD. The roles for this enzyme shall be fully explored in section 1.8. In the liver, the 5 α - and 5 β -reductase enzymes metabolise cortisol and cortisone through a series of reducing steps, to solubilise cortisol and cortisone for urinary excretion [White *et al.*, 1997] (Figure 1.3).

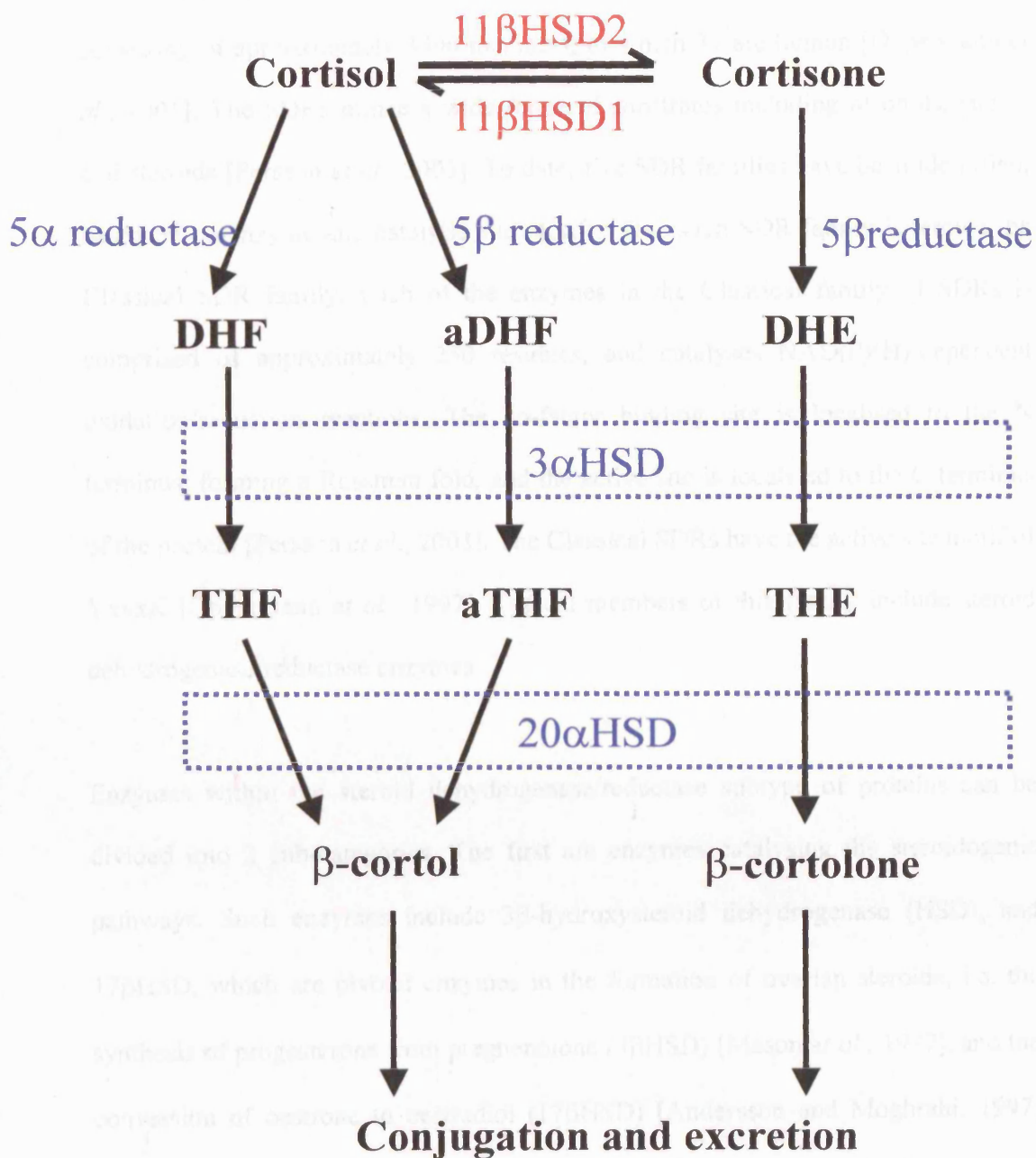


Figure 1.3: Mechanism by which cortisol and cortisone are metabolised in target tissue (red) and in the liver for excretion (blue). DHF=dihydrocortisol, aDHF=allo dihydrocortisol, DHE=dihydrocortisone, THF=tetrahydrocortisol, aTHF=allo tetrahydrocortisol, THE=tetrahydrocortisone. Adapted from White *et al.*, 1997

1.7 Short-chain dehydrogenases/reductas

The short-chain dehydrogenases/reductases (SDRs) are a superfamily of enzymes consisting of approximately 3000 members, of which 37 are human [Oppermann *et al.*, 2001]. The SDRs utilise a wide range of substrates including alcohols, sugars and steroids [Persson *et al.*, 2003]. To date, five SDR families have been identified, based on co-enzyme and catalytic site motifs. One such SDR family is termed the Classical SDR family. Each of the enzymes in the Classical family of SDRs is comprised of approximately 250 residues, and catalyses NAD(P)(H)-dependent oxidation/reduction reactions. The co-factor binding site is localised to the N terminus, forming a Rossmann fold, and the active site is localised to the C terminus of the protein [Persson *et al.*, 2003]. The Classical SDRs have the active site motif of YxxxK [Oppermann *et al.*, 1997]. Typical members of this family include steroid dehydrogenase/reductase enzymes

Enzymes within the steroid dehydrogenase/reductase subtype of proteins can be divided into 2 sub-categories. The first are enzymes catalysing the steroidogenic pathways. Such enzymes include 3 β -hydroxysteroid dehydrogenase (HSD), and 17 β HSD, which are pivotal enzymes in the formation of ovarian steroids; i.e. the synthesis of progesterone from pregnenolone (3 β HSD) [Mason *et al.*, 1997], and the conversion of oestrone to oestradiol (17 β HSD) [Andersson and Moghrabi, 1997] (see Section 1.2.2). Steroid metabolism serves 2 purposes. The first for the excretion of steroid through the generation of polar and /or charged steroid metabolites e.g., the conversion of progesterone to 20 α -dihydroprogesterone by 20 α HSD, and in metabolism of steroids within their target organs for regulating the potency of the

steroid at its receptor. This mechanism is integral for local control of steroid hormone activity. Such enzymes include the glucocorticoid metabolising enzyme, 11 β HSD which regulates the activity of cortisol, the active glucocorticoid, through interconversion with cortisone, its inactive keto-steroid metabolite [Walker and Stewart, 2003].

1.8 11 β HSD

As previously mentioned in section 1.6.4, in target tissues the active glucocorticoid cortisol is inter-converted to its inactive keto-steroid metabolite cortisone by isoforms of the enzyme 11 β HSD [Blum and Maser 2003; Walker and Stewart, 2003, Figure 1.4]. To date, 3 isoforms of 11 β HSD have been cloned. 11 β HSD1 and 11 β HSD2 have been extensively studied and functionally characterised. However, the functional properties of 11 β HSD3 are yet to be elucidated.

1.8.1. 11 β HSD1

11 β HSD1 was originally isolated from a rat cDNA library [Agarwal *et al.*, 1989]. It is a 34kDa protein with 2 hypothesised sites for N-glycosylation. Monder and Lakshmi [1990] localised 11 β HSD1 mRNA expression to the liver, lungs, testis, and kidney. Subsequent cloning of human 11 β HSD1 localised the gene to chromosome 1, with ubiquitous gene expression in most tissues including the ovary [Tannin *et al.*, 1991]. Kinetic analysis of 11 β HSD1 has shown that it is predominately a low affinity 11 ketosteroid reductase (11KSR) *in vivo*, with a micromolar affinity for cortisol ($K_m=27\mu M$), and a co-factor preference for NADP(H). Functional analysis

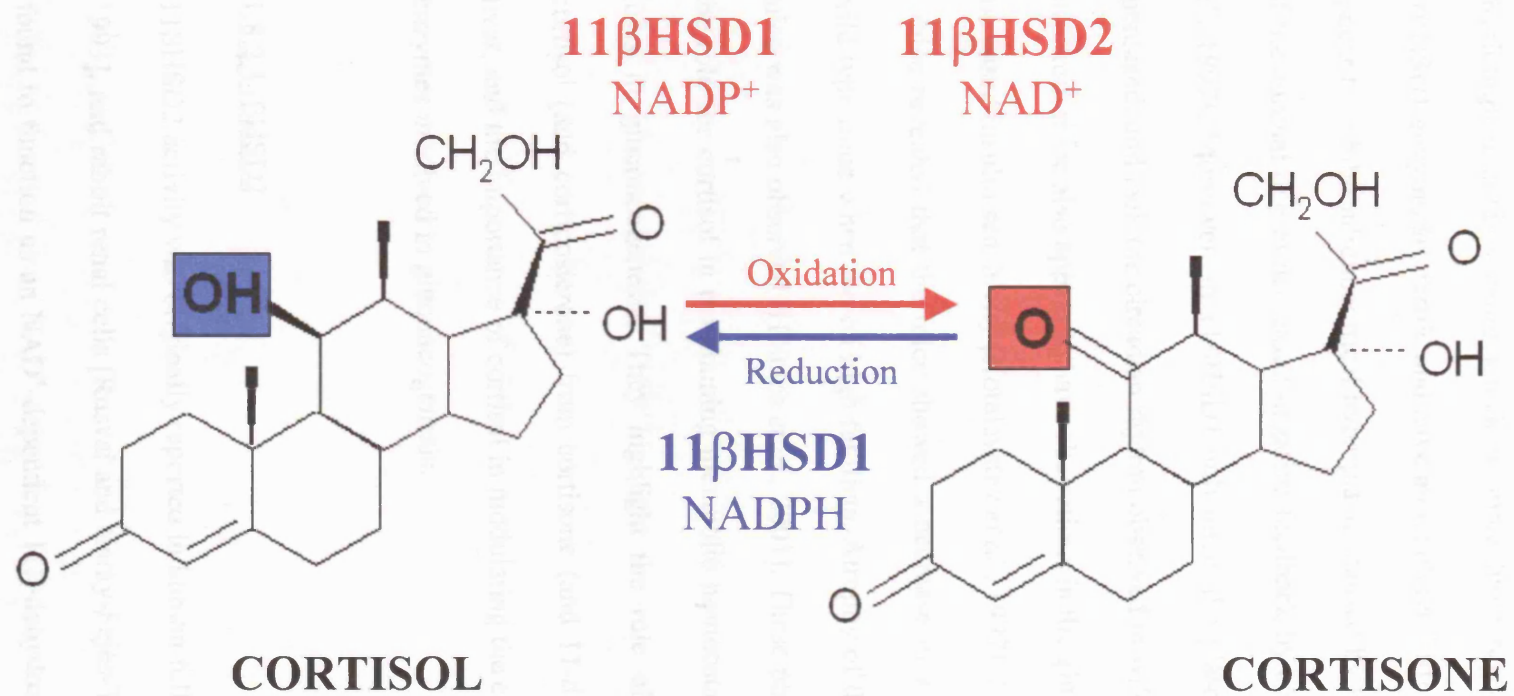


Figure 1.4: Inter-conversion of cortisol and cortisone by isoforms of the enzyme 11 β HSD. Highlighted in red is the oxidation of the C11 hydroxyl group of cortisol to a ketone to form cortisone, and in blue, the reduction of the C11 group to a hydroxyl to form cortisol.

revealed that 11 β HSD1 is a bi-directional enzyme *in vitro*, 11KSR enzyme with a basal dehydrogenase activity.

Physiological studies using knockout mice have revealed that mice lacking the 11 β HSD1 enzyme are fertile and have an unaltered birth weight and litter size. They appear to exhibit adrenocortical hyperplasia, caused by increased ACTH stimulation of the adrenal due to decreased negative feedback by corticosterone [Kotelevstev *et al.*, 1997]. Moreover, in 11 β HSD1 null mice, the plasma corticosterone levels are increased, and lack the circadian rhythm observed in wild-type mice. The 11 β HSD1 knockout mice also appear to have aberrations in the gluconeogenic pathway, and increased insulin sensitivity [Kotelevstev *et al.*, 1997]. Further analysis of 11 β HSD1^{-/-} mice revealed that the mice showed a decrease in weight gain in comparison to wild type mice when fed on high fat diets. Atrophy of the thymus of the 11 β HSD1^{-/-} mice was also observed (Holmes *et al.*, 2001]. These phenotypes are consistent with the role for cortisol in maintaining metabolic homeostasis, with specific functional roles in gluconeogenesis. They highlight the role of 11 β HSD1 in regenerating cortisol (and corticosterone) from cortisone (and 11-dehydrocorticosterone) in the liver, and the importance of cortisol in modulating the expression and activity of key enzymes involved in gluconeogenesis.

1.8.2 11 β HSD2

11 β HSD2 activity was originally reported in human full term placenta [Brown *et al.*, 1993], and rabbit renal cells [Rusvai and Naray-Fejes-Toth, 1993]. The enzyme was found to function as an NAD⁺-dependent 11 β -dehydrogenase converting cortisol to

cortisone, with a nanomolar affinity for cortisol ($K_m=60\text{nM}$) [Mercer and Krozowski, 1992; Brown *et al.*, 1993]. Subsequent studies cloned and characterised 11 β HSD2 from human and sheep kidney [Albiston *et al.*, 1994; Agarwal *et al.*, 1994]. Northern blotting revealed expression of 11 β HSD2 in tissues involved in water re-absorption including the placenta, distal nephron of the kidney, colon, parotid gland and ovary. As well as binding to the GR, cortisol and corticosterone can also bind to the mineralocorticoid receptor which have little inherent specificity for binding aldosterone. In these tissues 11 β HSD2 is thought to inactivate cortisol to cortisone, thus inhibiting promiscuous occupation of mineralocorticoid receptor by cortisol. Such occupation of the mineralocorticoid can result in the syndrome of 'apparent mineralocorticoid excess' (AME) [White, 2001]. 11 β HSD2 is also involved in regulating glucocorticoid availability to the foetus serving as a barrier mechanism in the placenta oxidising cortisol to cortisone. This mechanism exists to prevent foetal exposure to glucocorticoids, which would stimulate premature differentiation of foetal tissue, and result in intra-uterine growth retardation [reviewed by Michael *et al.*, 2003].

Phenotypic analysis of mice bearing a gene deletion for 11 β HSD2 revealed that 50% of 11 β HSD2 null mice died within 48 hours of birth [Kotelevtsev *et al.*, 1999]. Reasons for this included cardiac arrest due to severe hypokalaemia (low potassium) caused by hyper-activation of the mineralocorticoid receptors [Holmes *et al.*, 2001]. These symptoms are consistent with that of severe AME.

1.8.3 11 β HSD3

Over the past decade, several authors have suggested the existence of a third isoform of 11 β HSD [Gomez-Sanchez *et al.*, 1997; Ge *et al.*, 1997; Michael *et al.*, 1997; Ge *et al.*, 2000]. Recently the primary sequences for 11 β HSD3 from 6 individual species have been deposited in the NCBI protein database by Huang [<http://www.ncbi.nlm.nih.gov/entrez/query>]. To date, nothing is known of the tissue distribution, kinetic, structural and functional properties of this enzyme. However, personal communication with Huang has revealed that each of the 6 species 11 β HSD3 was isolated from brain, and the gene encoding 11 β HSD3 has also been cloned from human brain.

1.8.4 11 β HSD enzymes and Ovarian function

1.8.4.1 11 β HSD enzymes and their link to *in vitro* fertilisation-embryo transfer outcome

Publications over the last decade have suggested a possible link between cortisol-cortisone conversion in ovarian cells, and the outcome of *in vitro* fertilisation-embryo transfer (IVF-ET). The net rate of cortisol oxidation to cortisone has been shown to correlate inversely with the probability of conception by IVF-ET; a low 11 β HSD activity reflects a higher probability of achieving pregnancy in women undergoing IVF-ET [Michael *et al.*, 1993; 1995; 1999; Keay *et al.*, 2002; Thurston *et al.*, 2003a; Lewicka *et al.*, 2003].

There have been conflicting studies that have found no correlation between 11 β HSD activity and IVF-ET outcome [O'Shaughnessy *et al.*, 1997; Thomas *et al.*, 1998; Andersen *et al.*, 1999]. However, differences in granulosa cell culture times, and storage of granulosa cells in human FF, known to contain modulators of 11 β HSD activity could explain these differences [reviewed by Michael *et al.*, 2003].

Subsequent studies have isolated modulatory compounds of 11 β HSD activity in human FF. Studies by Thurston *et al.* [2002] first reported the presence of hydrophilic stimulators and hydrophobic inhibitors of 11 β HSD activity in human FF. The relative ratio of stimulatory compounds to inhibitory compounds, i.e. the presence of a low stimulatory activity, and a high inhibitory activity, has been related to the successful outcome of IVF-ET [Thurston *et al.*, 2003a].

1.8.4.2 Spatial and temporal expression patterns of 11 β HSD enzymes in the ovary

The expression of 11 β HSD enzyme isoforms has been documented in the human ovary. Immuno-histochemical studies have localised 11 β HSD1 expression to the oocyte, luteinising granulosa cells, theca cells and ovarian surface epithelial cells. The 11 β HSD2 isoform has been localised to theca, mural and cumulus granulosa cells from immature follicles [Ricketts *et al.*, 1998; Smith *et al.*, 2000; Yong *et al.*, 2000]. The pattern of 11 β HSD expression in the ovary is dependent on the stage of the ovarian cycle. In rat [Tetsuka *et al.*, 1999a] and human [Tetsuka *et al.*, 1997] granulosa cells, isolated from the pre-ovulatory stage of the ovarian cycle, 11 β HSD2 mRNA was detected. In rat and human granulosa cells undergoing luteinisation, an increase in 11 β HSD1 mRNA was detected following the administration of hCG.

This also coincided with a decrease in 11 β HSD2 mRNA expression. These results were further confirmed by 11 β HSD1 and 11 β HSD2 mRNA expression and activity studies in human granulosa-lutein cells, where only 11 β HSD1 mRNA was expressed [Michael *et al.*, 1997; Tetsuka *et al.*, 1997; Thurston *et al.*, 2003b]. The expression pattern of 11 β HSD isoforms has also been recently reported for the bovine ovary. Although the pattern of 11 β HSD1 expression changes less dramatically over the stages of the ovarian cycle, the follicular phase granulosa cells express predominantly 11 β HSD2 and luteal phase cells express predominantly 11 β HSD1 [Tetsuka *et al.*, 2003]

There are many possible reasons why the pattern of ovarian 11 β HSD expression changes throughout the ovarian cycle. In a wide range of teleost species, cortisol stimulates oocyte maturation [Kime *et al.*, 1992]. Hence, 11 β HSD1 may be required at ovulation to increase the local regeneration of glucocorticoids. In humans there is circumstantial evidence of a role of cortisol for oocyte maturation. In FF cortisol levels peak around the time of ovulation [Harlow *et al.*, 1997], and in women undergoing IVF-ET a positive link was found between oocyte maturity and cortisol levels in human FF [Fateh *et al.*, 1989]. It has also been suggested that intrafollicular concentrations of cortisol are important for the final stages of oogenesis. This is supported by immunocytochemical data showing the expression of high levels of 11 β HSD1 mRNA and protein in the rat oocyte [Benediktsson *et al.*, 1992]. Noteworthy is that transcription is blocked between pre-ovulatory meiosis and the mitotic division of the embryo to the 2-cell stage. Therefore high expression of 11 β HSD1 in the rat oocyte could reflect the need to regenerate active glucocorticoids

in the oocyte, zygote and/or early embryo stages of development [reviewed by Michael *et al.*, 2003]

As reviewed in sections 1.1 and 1.3, ovulation is an inflammatory event that involves an inflammatory cascade, initiated in response to gonadotrophins. These events result in follicular rupture and oocyte expulsion. Following ovulation, the follicular tissue undergoes rapid repair and remodelling. Therefore it has been proposed that up-regulation of 11 β HSD1 mRNA in granulosa cells, would facilitate an increase in intrafollicular cortisol following gonadotrophin stimulation. Moreover, it has been suggested that the increase in cortisol synthesis, provides an anti-inflammatory mechanism by which prostaglandin production is suppressed following the inhibition of PLA₂ and decreased PGHS-2 expression [Hillier and Tetsuka 1998].

1.8.5 Hormonal regulation of 11 β HSD

To date there are numerous factors that have been shown to regulate the activity and expression of 11 β HSD isoforms. These factors are summarised in Table 1.1 and include lipids, peptides, growth factors, transcription factors and pharmacological agents.

In the ovary, there have been limited studies on the regulation of 11 β HSD. The regulatory factors that have been explored include IL-1 [Tetsuka *et al.*, 1999b; Yong *et al.*, 2002], and LH [Tetsuka *et al.*, 1999b], both of which stimulate 11 β HSD1 gene

expression. The role of LH and IL-1 in the regulation of 11 β HSD will be fully explored in sections 1.8.5.2 and 1.8.5.3 respectively.

1.8.5.1 Prostaglandins and 11 β HSD

As previously described, prostaglandins have numerous regulatory roles in the ovary. PGE₂ and PGF_{2 α} have been shown to increase 11 β HSD1 activity in cultured chorionic trophoblast cells. Moreover, PGF_{2 α} can stimulate the reductase activity of 11 β HSD1 (in a concentration-dependent manner) at parturition in humans [Alfaidy *et al.*, 2001]. The mechanism is speculated to be via mobilisation of Ca²⁺, activation of PKC, and by phosphorylation of 11 β HSD1, mediated via the FP receptor [Alfaidy *et al.*, 2001]. In turn, cortisol has been shown paradoxically, to increase prostaglandin synthesis via the upregulation of PGHS2 expression and decreased prostaglandin metabolism via inhibition of 15-prostaglandin dehydrogenase (PGDH) in chorionic trophoblast cells [Alfaidy *et al.*, 2001; Challis *et al.*, 2002]. These data highlight the importance of the positive feedback loop between prostaglandins and cortisol in the onset of parturition in sheep and humans. In contrast to studies by Alfaidy *et al.*, [2001], PGE₂ and PGF_{2 α} have been shown to inhibit 11 β HSD2 in human choriocarcinoma JEG-3 cells [Hardy *et al.*, 2001].

Table 1.1: Regulatory compounds of 11 β HSD enzyme isoforms. ND = not determined by the study, - = inhibited activity or decreased expression, + = stimulated activity or increased expression, NS = not specified, NE = no effect.

Compound	11 β HSD isoform	Tissue	Activity	Expression	Reference
Lipids					
Cholesterol	NS	Rat kidney	-	ND	Buhler <i>et al.</i> , 1991
Chenodeoxycholic acid	11 β HSD1	Rat kidney	-	ND	Perschel <i>et al.</i> , 1991
Cortisol	11 β HSD1	Human adipocytes	ND	+ mRNA	Engeli <i>et al.</i> , 2004
Dexamethasone	11 β HSD1	Human osteoblasts	+	+ mRNA	Cooper <i>et al.</i> , 2002
	11 β HSD1	Term amnion fibroblasts	ND	+ mRNA	Sun and Myatt, 2003
DHEA	11 β HSD1	Rat liver cDNA	ND	-	Gu <i>et al.</i> , 2003
Glycyrrhetic acid	11 β HSD1	Rat liver microsomes	-	ND	Monder <i>et al.</i> , 1991
Lanosterol	NS	Rat kidney	-	ND	Buhler <i>et al.</i> , 1991
Leukotriene B ₄	11 β HSD2	JEG3 cells	-	NE	Hardy <i>et al.</i> , 1999
Lithocholic acid	11 β HSD1	Rat kidney	-	ND	Perschel <i>et al.</i> , 1991
Medroxyprogesterone	11 β HSD2	Ishikawa cells	+	ND	Darnel <i>et al.</i> , 1999
Oestradiol	11 β HSD2	Human placental trophoblast	-	ND	Sun <i>et al.</i> , 1998
	11 β HSD2	Ishikawa cells	+	+ mRNA and protein	Darnel <i>et al.</i> , 1999
	11 β HSD2	Rat kidney	NE	- mRNA and protein	Gomez-Sanchez <i>et al.</i> , 2003
	11 β HSD1	Rat kidney	-	ND	Gomez-Sanchez <i>et al.</i> , 2003
PGE ₂	11 β HSD2	JEG3 cells	-	NE	Hardy <i>et al.</i> , 1999
PGF _{2α}	11 β HSD2	Human placental microsomes	+	ND	Hardy <i>et al.</i> , 2001
PGF _{2α}	11 β HSD1	Human chorionic trophoblast	+	ND	Alfaidy <i>et al.</i> , 2001
Progesterone	11 β HSD2	Human placental trophoblast	-	- mRNA	Sun <i>et al.</i> , 1998
Testosterone	11 β HSD1	Rat Leydig cells	-	ND	Gao <i>et al.</i> , 1997a

<u>Growth Factors</u>					
EGF	11 β HSD1	Rat Leydig cells	-	ND	Gao <i>et al.</i> , 1997a
	11 β HSD1	Rat Leydig cells	+	ND	Gao <i>et al.</i> , 1997a
	11 β HSD2	Ishikawa cells	-	ND	Darnel <i>et al.</i> , 1999
IGF-I (GH activated)	11 β HSD1	Human omental adipose	-	ND	Moore <i>et al.</i> , 1999
<u>Cytokines</u>					
IL-1 β	11 β HSD1	Glomerular mesangial cells	+	ND	Escher <i>et al.</i> , 1997
		Rat granulosa cells	ND	+ mRNA	Tetsuka <i>et al.</i> , 1999b
		hOSE cells	+	ND	Yong <i>et al.</i> , 2002
		Human adipose stromal cells	+	+ mRNA	Tomlinson <i>et al.</i> , 2001
		MG-63 cells	+	+ mRNA	Friedberg <i>et al.</i> , 2003, Cooper <i>et al.</i> , 2001
IL-4	11 β HSD1	Differentiating monocytes	+	+	Thieringer <i>et al.</i> , 2001
IL-4 +INF γ	11 β HSD1	Human granulosa lutein cell	+	ND	Evagelatou <i>et al.</i> , 1997
IL-5	11 β HSD1	Human granulosa lutein cell	+	ND	Evagelatou <i>et al.</i> , 1997
IL-6	11 β HSD1	Human granulosa lutein cell	+	ND	Evagelatou <i>et al.</i> , 1997
IL-13	11 β HSD1	Differentiating monocytes	+	+	Thieringer <i>et al.</i> , 2001
Leukocytes	11 β HSD1	Human granulosa lutein cell	+	ND	Evagelatou <i>et al.</i> , 1997
TNF α	11 β HSD1	Human adipose stromal cells	+	+ mRNA	Tomlinson <i>et al.</i> , 2001
	11 β HSD1	Human adipocytes	+	+ mRNA	Friedberg <i>et al.</i> , 2003
	11 β HSD2	LLC-PK	-	- mRNA	Heiniger <i>et al.</i> , 2001
<u>Peptide hormones</u>					
ACTH	11 β HSD1	Human adipocytes	-	NE	Freidberg <i>et al.</i> , 2003
CRH	11 β HSD1	Human adipocytes	-	NE	Freidberg <i>et al.</i> , 2003
LHRH	11 β HSD1	Rat granulosa cells	ND	+ mRNA	Tetsuka <i>et al.</i> , 1999b
Noradrenaline	11 β HSD2	Placental trophoblasts		-	Sarker <i>et al.</i> , 2001
rhFSH	11 β HSD1	Rat granulosa cells	ND	+ mRNA	Tetsuka <i>et al.</i> , 1999b

rhLH	11 β HSD1	Rat granulosa cells	ND	+ mRNA	Tetsuka <i>et al.</i> , 1999b
<u>Modifiers of Signalling Transduction</u>					
Carbenoxolone	11 β HSD1	Human	-	ND	Stewart <i>et al.</i> , 1990
Calcium	11 β HSD2	Human placental microsomes	-	ND	Hardy <i>et al.</i> , 2001
C/EBP α	11 β HSD1	Hepatoma cells	ND	+ mRNA	Williams <i>et al.</i> , 2000
C/EPB β	11 β HSD1	Hepatoma cells	ND	+ mRNA	Williams <i>et al.</i> , 2000
Forskolin	11 β HSD1	Rat granulosa cells	ND	+ mRNA	Tetsuka <i>et al.</i> , 1999b
	11 β HSD2	Human placental chorion,	+	+ mRNA	Sun <i>et al.</i> , 1998
Hypoxia via Erg-1	11 β HSD2	LLC-PK1 cells	ND	- mRNA	Heiniger <i>et al.</i> , 2003
Indomethacin	11 β HSD2	JEG3 cells	-	ND	Hardy <i>et al.</i> , 1999
LXR	11 β HSD1	3T3-L1 cells	-	- mRNA	Stulnig <i>et al.</i> , 2002
MAPK ERK inhibitor	11 β HSD2	LLC-PK	+	ND	Heiniger <i>et al.</i> , 2001
MAPK p38 inhibitor	11 β HSD2	LLC-PK	+	ND	Heiniger <i>et al.</i> , 2001
PMA	11 β HSD1	Rat granulosa cells	ND	+ mRNA	Tetsuka <i>et al.</i> , 1999b
	11 β HSD2	LLC-PK	-	- mRNA	Heiniger <i>et al.</i> , 2001
PPAR γ ligand-thiazolidinedione	11 β HSD1	3T3-L1 cells	-	- mRNA	Berger <i>et al.</i> , 2001
RU486	11 β HSD1	Term amnion fibroblasts	ND	- mRNA	Sun and Myatt, 2003

1.8.4.2 LH and 11 β HSD

In reproductive tissues, LH and hCG exert differential effects on 11 β HSD1 activity and expression. Studies have shown that in leydig cells increased LH can increase 11 β HSD activity [Gao *et al.*, 1997]. The role of corticosterone in the testis is speculated to be in suppressing the circulating and local concentrations of

testosterone. Studies comparing the oxidative activity of 11 β HSD in dominant and subordinate male rats have shown that the dominant rat has an increased 11 β HSD oxidative activity. This is thought to be due to an increase in LH, which increases 11 β HSD oxidative activity, allowing the Leydig cells to produce testosterone, and for the dominant rat to maintain its fertility despite higher levels of corticosterone [Hardy *et al.*, 2002].

Measurements of free cortisol in follicular fluid following the LH surge have shown a dramatic increase in free and total cortisol [Harlow *et al.*, 1997]. 11 β HSD1 mRNA expression in the rat ovary has been shown to be regulated LH. Moreover, LH was found to act in a concentration-dependent manner to increase 11 β HSD1 mRNA expression in rat granulosa cells. This effect was found to be additive when rat granulosa cells were co-treated with IL-1 β [Tetsuka *et al.*, 1999b]. Hence, the increased expression of 11 β HSD1 in response to LH could contribute to the increase in intrafollicular cortisol concentrations following the LH surge.

1.8.5.3 IL-1 and 11 β HSD

The roles for inflammatory cytokines in the regulation of 11 β HSD activity and expression have been demonstrated in several tissue types. In adipose tissue, osteoblasts, placenta and the ovary, IL-1 β induces the expression of 11 β HSD1 mRNA. In the rat ovary, Tetsuka *et al.*, [1999b] have shown that IL-1 β can up-regulate 11 β HSD1 mRNA expression in a concentration-dependent manner. In other studies using human ovarian surface epithelial cells (hOSE), IL-1 α was shown to

increase 11 β HSD1 mRNA expression and the 11KSR activity of 11 β HSD1 [Yong *et al.*, 2002]. A recent publications by Rae *et al.*, [2004] has also shown that IL-1 α can induce 11 β HSD1 mRNA expression. Moreover, IL-1 α also increased the expression of the GR α subtype, highlighting a possible mechanism by which IL-1 α may stimulate the anti-inflammatory activities of cortisol through the up-regulation of the GR α gene coincident with increased reactivation of cortisol in target cells [Rae *et al.*, 2004].

1.9 Project aims and justification

The cellular mechanisms that control cortisol metabolism in the ovary still remain to be elucidated. Prostaglandins have been extensively implicated in the regulation of ovarian function, specifically within ovulation and CL dynamics. However, their precise mechanisms of action within the human ovary still remain unclear. Although cortisol metabolism is thought to be regulated by prostaglandins in several reproductive tissues, the cellular mechanisms of control are yet to be investigated in the ovary.

Therefore the aims of this study were:

- a) To determine the modulatory properties of human FF on 11 β HSD1 activity and expression, and to determine the identity of the modulatory compounds
- b) To investigate the effect of progesterone on the activity and expression of 11 β HSD1 in human granulosa-lutein cells
- c) To elucidate the effects of prostaglandins on basal 11 β HSD1 activity and expression in human granulosa-lutein cells

- d) To elucidate the effects of hCG and IL-1 β on 11 β HSD1 activity and expression in human granulosa-lutein cells, and the possible mediatory role for eicosanoids in any responses to hCG and /or IL-1 β
- e) To predict the topology and tertiary structures of 11 β HSD1 as these relate to the regulation of enzyme activities by hydrophilic and hydrophobic hormones using current literature, and *in silico* approaches.

Chapter Two: Materials and Methods

Chapter 2- Materials and Methods

2.1 Chemicals and Reagents

PGE₂ and PGF_{2α} antibodies were kindly donated by Drs. R.W Kelly and N.L. Poyser respectively (University of Edinburgh, UK). Progesterone antibody was obtained from the Central Veterinary Laboratory (Weybridge, Surrey, UK). hCG was a kind gift from the National Hormone and Peptide Program-NIDDK (California, USA).

[7(n)-³H]-cholesterol (8Ci/mmol), [1,2,6,7-³H]-cortisol (60Ci/mmol), [1,2(n)-³H]-cortisone (45Ci/mmol), [2,4,6,7-³H]-oestradiol (81Ci/mmol), [5,6,8,11,12,14,15-³H]-PGE₂ (164Ci/mmol), [5,6,8,9,11,12,14,15-³H]-PGF_{2α} (219Ci/mmol), [1,2,6,7-³H]-progesterone (86Ci/mmol), [1,2,6,7-³H]-testosterone (70Ci/mmol), Dextran T500, ECL™ Western blotting detection reagents, PD-10 columns, and Hyperfilm™ ECL™ were each obtained from Amersham Biosciences, UK Ltd (Little Chalfont, Bucks., UK). [7-³H]-pregnenolone (14Ci/mmol) was purchased from Perkin Elmer, (Beaconsfield, Bucks.,UK).

β-mercaptoethanol, charcoal, chloroform, di-sodium hydrogen orthophosphate 12-hydrate (Na₂HPO₄.12H₂O), ethanol, ethyl acetate, gelatin (powder), methanol, sodium di-hydrogen orthophosphate dihydrate (NaH₂PO₄.2H₂O), and ultra-pure water were supplied by BDH (Poole, Dorset, UK).

1:1 Dulbecco's modified Eagle's medium:Hams's F₁₂ (DMEM:Ham's F₁₂) medium, foetal calf serum (FCS), L-glutamine, penicillin/streptomycin and Dulbecco's

phosphate-buffered saline (PBS) were purchased from Life technologies Ltd (Gibco BRL, Paisley, UK). TLC aluminium sheets (20cmx20cm, Silica gel 60) were supplied by Merck (Poole, Dorset, UK) and Ultima-Gold scintillant was purchased from Packard Biosciences B.V (Pangbourne, Berks, UK).

Bicinchoninic acid (BCA) protein assay reagent A (Na_2CO_3 , NaHCO_3 , $\text{C}_4\text{H}_4\text{O}_6\text{Na}_2$ in 0.2% N NaOH and BCA detection reagent), BCA reagent B (4% (w/v) CuSO_4) and Bovine serum albumin (BSA) protein standard (2mg/ml) were each obtained from Pierce Science UK Ltd (Tattenhall, Cheshire, UK). Extra thick filter paper and Tris-electrophoresis grade were supplied by Bio-Rad (Hercules, California, USA). Polyvinylidene difluoride (PVDF) (Immobilon™-P transfer) membrane was purchased from Millipore (Watford, England). 30% (w/v) polyacrylamide (Ultra pure Protogel®) and X10 Tris-glycine-SDS electrophoresis grade running buffer were purchased from National diagnostics (Atlanta, Georgia, USA).

Sheep anti-human 11 β HSD1 primary monoclonal antibody (IgG fraction) and mouse anti-human GAPDH primary monoclonal antibody (IgG fraction) were both supplied by The Binding site Ltd. (Birmingham, UK). Donkey anti-sheep peroxidase conjugated IgG and rabbit anti-mouse peroxidase conjugated IgG were purchased from Sigma-Aldrich (Poole, Dorset, UK). NS398 was obtained from Cayman Chemicals (Alexis Platform, Nottingham, UK). Protease inhibitor Cocktail Set I (containing 500 μM 4-(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF), 150nM aprotinin, 1 μM E-64, 0.5mM ethylene-diaminetetraacetic acid (EDTA), and 1 μM leupeptin) were obtained from Calbiochem®, c/o Merck Bioscience Ltd (Beeston,

Nottingham, UK). SeeBlue[®] Plus 2 Pre-stained Protein standard molecular weight markers, range 250-4 kDa were supplied by Invitrogen Life Technologies (Paisley, UK). GBX fixer and replenisher, and GBX developer and replenisher were supplied by Eastman Kodak Company (Rochester, New York, USA).

All other chemicals and solvents were supplied by Sigma-Aldrich (Poole, Dorset, UK).

2.2 Collection and storage of follicular aspirates

2.2.1 Patient samples

Ovarian follicular aspirates containing granulosa cells were collected from women undergoing assisted conception at the Lister Hospital, Chelsea Bridge Road, London, UK. Collections were carried out with approval of the local ethics committee, with informed patient consent, in accordance with the Declaration of Helsinki and following HFEA guidelines.

Pituitary down-regulation was achieved by administration of a GnRH analogue, Suprecur (Shire Pharmaceuticals, Andover, Hants, UK), 500µg per day from day 2 of the ovarian cycle for 10-21 days. Administration of the GnRH analogue was then continued in conjunction with purified urinary human menopausal gonadotrophin, Menogon (Ferring Pharmaceuticals, Feltham, Middlesex, UK), 2-4 ampoules daily for 10-14 days, followed by a single intramuscular injection of hCG, Profasi (Serono, Welwyn Garden City, Herts, UK), 5,000-10,000IU administered 36 hours

prior to oocyte retrieval. Follicles were subsequently aspirated transvaginally under local anaesthesia.

2.2.2 Isolation of human granulosa cells

Human granulosa cells were isolated following the method previously described by Webley *et al.* [1988]. Granulosa cells and contaminating blood cells from a single patient were separated from the supernatant follicular fluid and follicular flushing medium by centrifugation at 250g for 10 minutes at 4°C, with no brake, in a Beckman GS-6R centrifuge. The supernatant was discarded and the cell pellet resuspended in 10ml Dulbecco's PBS. The cell suspension was overlaid onto 10ml 60% (v/v) Percoll. The Percoll gradients were centrifuged at 1000g for 20 minutes at 4°C with no brake. This resulted in sedimentation of erythrocytes at the bottom of the tube, with the granulosa cells settling on the Percoll-PBS interface. Granulosa cells were aspirated from the interface, resuspended in 10ml PBS, and centrifuged at 250g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 10ml PBS. This wash step was repeated a total of three times, and in the final wash step, cells from a single patient were pooled together.

Following the final wash step, the cell pellet was resuspended in 1ml PBS, and a 10µl aliquot was mixed with 0.4% (w/v) trypan blue solution to assess cell viability. Viable granulosa cells did not take up the trypan blue stain, and were counted under the microscope (magnification x200) using a haemocytometer. After dilution the cells were cultured in 1:1 (v/v) DMEM:Ham's F₁₂ medium supplemented with 10% (v/v) FCS, penicillin (87,000 IU/L), streptomycin (87 mg/L) and 2mM L-glutamine.

For studies of 11 β HSD activity and progesterone secretion, cells were seeded into sterile 24-well plates at a density of 5×10^4 cells/ml of culture medium, with a volume of 1ml/well. For studies of prostaglandin secretion, cells were seeded into sterile 24-well plates at a density of 1×10^5 cells/ml of culture medium, with a volume of 1ml/well. For experiments to determine effects on protein expression, cells were seeded into a sterile 6 well plate at a density of 5×10^5 cells/ml of culture medium, with a volume of 2ml/well. Cells were subsequently incubated for 2 days at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air to allow cells to attach to the well of the cell culture plates. At the end of the 2 day incubation, the medium was aspirated from the wells and discarded. The cells were rinsed with approximately 200 μ l pre-warmed serum-free medium to remove any residual serum. The washing medium was aspirated, discarded, and serum-free medium and treatments were added to a total volume of 1 or 2ml depending on cell culture plates. When using serum-free medium, bovine serum albumin was not added since this binds to cortisol with high affinity and would thus be a confounding factor in quantifying 11 β HSD activities. Cells were subsequently incubated at 37°C as described above for a period of 0 to 24 hours, depending on the experimental design.

2.3 Radioimmunoassays

2.3.1 Progesterone RIA

Progesterone concentrations were determined by RIA as previously described by Pallikaros *et al.* [1995]. A progesterone standard curve was prepared from a 1 μ g/ml stock solution of progesterone in ethanol, stored at -20°C. A volume of 10 μ l

progesterone standard was transferred to a borosilicate tube, and the ethanol was evaporated under nitrogen. The progesterone was resuspended in 1ml serum-free medium to produce the top standard progesterone concentration of 10ng/ml (31.80nM). The top standard was double diluted in medium to produce the following standards: 15.90, 7.95, 3.98, 1.99, 0.99, 0.50, and 0.25nM progesterone. Volumes of 100µl of each standard were aliquoted in triplicate and 100µl serum-free medium were aliquoted into the 0nM progesterone (B_0), total and non-specific binding (NSB) tubes in triplicate. The unknown samples were serially diluted in serum-free medium into a total volume of 100µl. The unknown samples were diluted in serum-free medium between 1/100 to 1/200 into a total diluted sample volume of 100µl per tube.

Progesterone antiserum was diluted 1/100 (v/v) in phosphate azide saline-gelatin (PAS-gel) buffer and stored in 100µl aliquots at -20°C. Immediately prior to use, a 100µl aliquot was thawed and further diluted 1/100 (v/v) in PAS-gel buffer resulting in a working antibody dilution of 1/10,000 (v/v). A 100µl volume of diluted antibody was added to each standard and sample tube, excluding those for the determination of total and NSB radioactivity where 100µl PAS-gel buffer was substituted in place of antibody.

[1,2,6,7-³H]-progesterone was diluted in PAS-gel buffer to give the required 10,000cpm per 100µl. A volume of 100µl of diluted [1,2,6,7-³H]-progesterone was added to all assay tubes. Each tube was vortexed, covered in aluminium foil, and incubated at 4°C overnight.

The next day, a solution of 0.025% (w/v) dextran was prepared in PAS-gel buffer. After the dextran had dissolved, 0.25% (w/v) charcoal was added and mixed for 30 minutes at 4°C. A volume of 500µl activated dextran-coated charcoal was added to each assay tube, with the exception of the total radioactivity tubes, which received 500µl PAS-gel buffer. The tubes were vortexed and centrifuged for 10 minutes at 1000g at 4°C with no brake to precipitate unbound progesterone. The supernatants were decanted into mini-scintillation vials, and 2ml Ultima-Gold scintillant was added into each vial, which were subsequently capped and vortexed. Radioactivity in cpm was measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter, with a count time of 1 minute per vial. The counts were analysed using a DOS-RIA software program using the logit % B/B₀ whereby the data from the standard curve was linearised. Intra and inter-assay coefficients of variation were 9% (n=15) and 14% (n=40) respectively at 30% binding.

2.3.2 PGE₂ RIA

PGE₂ concentrations were determined as previously described by Poyser [1987]. A PGE₂ standard curve was prepared from a 1µg/ml stock solution of PGE₂ in ethanol, stored at -20°C. A 10µl volume of PGE₂ stock was transferred to a borosilicate tube, evaporated to dryness under nitrogen, and resuspended in 100µl serum-free medium to produce a stock of 100ng/ml PGE₂. A 50µl aliquot of the 100ng/ml stock was diluted in serum-free medium to a total volume of 1ml to generate the top standard concentration of 5ng/ml (14.19nM) PGE₂. The top standard was double diluted in serum-free medium to generate the following standards: 7.09, 3.55, 1.77, 0.89, 0.44,

0.22, 0.11nM PGE₂. Volumes of 100µl of each standard were aliquoted in triplicate and 100µl serum-free medium were substituted into the B₀, total radioactivity, and NSB tubes. The unknown samples were either diluted 1/5 (v/v) in serum-free medium or assayed neat, with a total diluted sample volume of 100µl per tube. PGE₂ rabbit antiserum was stored in 50µl aliquots at -20°C. A 50µl aliquot of antiserum was diluted 1/100 (v/v) in phosphate buffer and stored in 500µl aliquots at -20°C. Immediately prior to assay, a 500µl aliquot of 1/100 antibody was thawed and further diluted 1/30 (v/v) in phosphate buffer resulting in a working dilution of 1/3,000 (v/v). A 100µl volume of diluted antibody was added to each standard and sample tube, excluding those for the determination of total and NSB radioactivity where 100µl of Tris buffer was substituted for the antibody. Stock [5,6,8,11,12,14,15-³H]-PGE₂ was diluted in Tris buffer to give the required 10,000cpm per 100µl. A volume of 100µl of the diluted [5,6,8,11,12,14,15-³H]-PGE₂ was added into all standard and unknown sample tubes. Each tube was vortexed, covered in aluminium foil, and incubated at 4°C overnight.

The next day, a solution of 0.1% (w/v) dextran was prepared in Tris buffer. After the dextran had dissolved, 1% (w/v) charcoal was added and mixed for 30 minutes at 4°C. A volume of 200µl activated dextran-coated charcoal was added to each assay tube, with the exception of the total radioactivity tubes, which received 200µl Tris buffer. The tubes were vortexed and centrifuged for 10 minutes at 1000g at 4°C with no brake to precipitate unbound PGE₂. The supernatants were decanted into miniscintillation vials, and 2ml Ultima-Gold scintillant was added into each vial, which were subsequently capped and vortexed. Radioactivity in cpm was measured on a

Beckman Coulter LS 6500 Multipurpose Scintillation counter, with a count time of 1 minute per vial. The counts were analysed using a DOS-RIA software program using the logit % B/B₀ whereby the data from the standard curve was linearised. Intra- and inter-assay co-efficients of variation were 10% (n=15) and 16% (n=28) respectively at 40% binding.

2.3.3 PGF_{2α} RIA

PGF_{2α} concentrations were determined as previously described by Kelly *et al.*, [1986]. A PGF_{2α} standard curve was prepared from a 1μg/ml stock solution of PGF_{2α} in ethanol, stored at -20°C. A 10μl volume of PGF_{2α} stock was transferred to a borosilicate tube, evaporated to dryness under nitrogen, and resuspended in 100μl serum-free medium to produce a stock of 100ng/ml PGF_{2α}. A 25μl aliquot of the 100ng/ml stock was diluted in serum-free medium to a total volume of 1ml to generate the top standard concentration of 2.5ng/ml (7.09nM) PGF_{2α}. The top standard was double diluted to generate the following standards: 3.55, 1.77, 0.89, 0.44, 0.22, 0.11, 0.06nM PGF_{2α}. Volumes of 100μl were aliquoted in triplicate and 100μl of serum-free medium were aliquoted into B₀, total radioactivity and NSB tubes. The unknown samples were either diluted 1/5 (v/v) in serum-free medium or assayed neat, with a total diluted sample volume of 100μl per tube.

PGF_{2α} rabbit antiserum was stored in 100μl aliquots at -20°C. A 100μl aliquot of antiserum was diluted 1/100 (v/v) in Tris buffer and stored in 100μl aliquots at

-20°C. Immediately prior to assay, a 100µl aliquot of 1/100 antibody was thawed and further diluted 1/100 (v/v) in Tris buffer resulting in a working dilution of 1/10,000 (v/v). A 100µl volume of diluted antibody was added to each standard and sample tube, excluding those for the determination of total and NSB radioactivity where 100µl of Tris buffer was substituted for the antibody. Stock [5,8,9,11,12,14,15-³H]-PGF_{2α} was diluted in Tris buffer to give the required 10,000cpm per 100µl. A volume of 100µl of the diluted [5,8,11,12,14,15-³H]-PGF_{2α} was added into all standard and unknown sample tubes. Each tube was vortexed, covered in aluminium foil, and incubated at 4°C overnight.

The next day, a solution of 0.1% (w/v) dextran was prepared in Tris buffer. After the dextran had dissolved, 1% (w/v) charcoal was added and mixed for 30 minutes at 4°C. A volume of 200µl activated dextran-coated charcoal was added to each assay tube, with the exception of the total radioactivity tubes, which received 200µl Tris buffer. The tubes were vortexed and centrifuged for 10 minutes at 1000g at 4°C with no brake to precipitate unbound PGF_{2α}. The supernatants were decanted into mini-scintillation vials, and 2ml Ultima-Gold scintillant was added into each vial, which were subsequently capped and vortexed. Radioactivity in cpm was measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter, with a count time of 1 minute per vial. The counts were analysed using a DOS-RIA software program using the logit % B/B₀ whereby the data from the standard curve was linearised. Intra- and inter- assay co-efficients of variation were 10% (n=15) and 14% (n=15) respectively, at 37% binding.

2.4 11 β HSD Assay

Human granulosa cells were isolated as described in section 2.2 and cultured at a density of 5×10^4 cells/ml culture medium, with a volume of 1ml medium/well in a 24 well culture plate. Cells were cultured for 2 days in 1:1 (v/v) DMEM:Ham's F₁₂ medium supplemented with 10% (v/v) FCS, penicillin (87,000 IU/L), streptomycin (87 mg/L) and 2mM L-glutamine, at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air.

At the end of the 2 day pre-incubation period, the serum-supplemented medium was aspirated from the wells and discarded. The cells were rinsed using approximately 200 μ l warmed serum-free medium to remove any residual serum and the washing medium was discarded. Cells were then transferred to pre-warmed serum-free medium supplemented with penicillin (87,000 IU/L), streptomycin (87 mg/L) and 2mM L-glutamine and incubation continued for a further 24 hours. When assessing the effects of chronic exposure to treatments, these treatments were added at 48 hours and were present throughout the third day of culture (i.e., from 48 to 72 hours). When assessing the effects of acute exposure to treatments, these treatments were added for the final 4 hours of incubation (i.e., from 68 to 72 hours). Where the purpose of the study was to assess the effects of treatments on 11 β HSD activities, 100 μ l of serum-free medium containing either 100pmol of either [1,2,6,7-³H]-cortisol (5nCi/pmol) or [1,2,(n)-³H]-cortisone (5nCi/pmol) were added to each well, with a final substrate concentration of 100nM. Cortisol-cortisone inter-conversion was measured over final 4 hours of incubation (i.e., from 68 to 72 hours). The

rationale to this approach was to ensure that all cells, irrespective of the treatment group and duration of treatment, were at the same stage of differentiation.

At the end of the 4-hour assay incubation period, the medium from each well was transferred into screw cap borosilicate tubes to which 2ml of ice cold chloroform was added. The tubes were then capped, vortexed, and subsequently centrifuged at 1000g for 20 minutes at 4°C. Following centrifugation, the aqueous phase was removed immediately and discarded. The samples were then evaporated to dryness in a Techne Dri-Block DB-3 sample concentrator at 45°C under nitrogen, before being stored at 4°C for up to 7 days pending resolution of steroids by thin layer chromatography (TLC).

Following evaporation to dryness, steroid residues were resuspended in 30µl ethyl acetate containing 1mM cortisol and 1mM cortisone. The tubes were vortexed, and 20µl volumes from each tube were transferred to individual lanes of a TLC plate. TLC plates were developed in an atmosphere of 92:8 (v/v) chloroform:95% (v/v) ethanol. The bands containing cortisol and cortisone were visualised by absorption of UV light at 254nm. The levels of radioactivity in the cortisol and cortisone regions were quantified using a Bioscan System 200 radiochromatogramme scanner (Lablogic, Sheffield, UK), with each lane counted for 5 minutes. The cortisol-cortisone inter-conversion was calculated by multiplication of percentage conversion of [1,2,6,7-³H]-cortisol to [1,2,6,7-³H]-cortisone (for 11β dehydrogenase activity) and [1,2,(n)³H]-cortisone to [1,2,(n)-³H]-cortisol (for 11-ketosteroid reductase

activity) by the total amount of steroid that was present at the beginning of the assay (100pmol/well).

2.5 Reverse Phase C18 column chromatography

C18 column chromatography was carried out as described by Morris *et al.*, [1992] using C18 sepak cartridges. Before use of each C18 cartridge, the cartridge was primed using 10ml methanol and then rinsed with 10ml distilled H₂O. Human follicular fluid (1ml) was then loaded onto a C18 cartridge and the loading eluent collected. Fractions were then sequentially eluted, based on hydrophobicity, using increasing concentrations of methanol in 1ml volumes (0, 10, 20, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100% (v/v)) methanol in distilled water. Each individual fraction generated was collected into a separate borosilicate glass tube. With the exception of 0-20% (v/v) methanol fractions, each tube was evaporated to dryness using a Techne Dri-Block DB-3 sample concentrator at 45°C under nitrogen, and re-suspended in 1ml volumes of 20% (v/v) methanol. Each tube was stored at -20°C pending analysis.

Pooled follicular fluid from large antral follicles was aliquoted into 8 volumes of 1ml. Each aliquot was spiked by the addition of [7(n)-³H]-cholesterol, [1,2,6,7-³H]-cortisol, [1,2,(n)-³H]-cortisone, [2,4,6,7-³H]-oestradiol, [5,6,8,11,12,14,15-³H]-PGE₂, [5,6,8,9,11,12,14,15-³H]-PGF_{2α}, [7-³H]-pregnenolone, [1,2,6,7-³H]-progesterone, or [7(n)-³H]-testosterone. The spiked samples were each loaded onto a separate C18 cartridge for fractionation by a stepwise methanol gradient as described above. Triplicate 100μl aliquots of each fraction generated were transferred to

scintillant vials containing 2ml Ultima gold scintillation and the amount of radioactivity quantified using a Beckman Coulter LS 6500 Multipurpose Scintillation counter with a count time of 1 minute per tube.

2.6 Assessment of 11 β HSD enzyme modulators in FF

Effects of FF fractions on 11 β HSD activity were assessed using a rat kidney homogenate as a source of both 11 β HSD1 and 11 β HSD2 enzymes [Thurston *et al.*, 2002].

Male Sprague-Dawley rats were sacrificed by terminal phenobarbitone anaesthesia [Marks *et al.*, 2003]. Kidneys were dissected and stored at -20°C. 0.5g rat kidney was added to 17ml hypotonic Tris-EDTA lysis buffer, and homogenised using a glass homogeniser. Restoration of isotonicity was achieved by the addition of 2ml 1.5mM potassium chloride to yield a renal tissue homogenate concentration of 25mg/ml lysis buffer [Sewell *et al.*, 1998]. The homogenate was centrifuged at 1000g for 20 minutes at 4°C. The supernatant was removed and from the supernatant, 100 μ l volumes were transferred to borosilicate screw cap tubes. Following this, 600 μ l PBS were added to each tube. Into each set of triplicate tubes, 100 μ l of each follicular fluid fraction was also added. For control tubes, 100 μ l of 20% (v/v) methanol was added instead of FF. Samples were pre-incubated for 30 minutes at 37°C in a shaking water-bath. To assess cortisol-cortisone interconversion by the NADP(H)-dependent 11 β HSD1 enzyme, either 100 μ l containing 100pmol [1,2,6,7-³H]-cortisol (5nCi/pmol) and 100 μ l 4mM NADP⁺ were added to each tube, or [1,2,(n)-³H]-cortisone (5nCi/pmol) and 4mM NADPH. To assess the effects on

11 β HSD2, this protocol was repeated using 4mM NAD⁺ in place of NADP⁺. Following this, the tubes were incubated for 1 hour at 37°C in a shaking water-bath. After 1 hour, the steroids were chloroform extracted, resolved by TLC and quantified using a Bioscan System 200 Radiochromatogramme Scanner as described in section 2.4.

2.7 Cell isolation and protein assay for Western blot

Cell isolation and protein assays were carried out as previously described by Thurston *et al* [2003c]. Following granulosa cell culture and treatment, spent medium was aspirated from the 6 well plates, and the cells were washed with 1ml ice-cold PBS. The PBS was aspirated, and cells were lysed in protein lysis buffer (119 μ l per well) containing protease inhibitor cocktail (see section 2.1 for cocktail recipe). Cells were freeze-thawed, removed from the 6 well plates by scraping, and aliquoted into Eppendorf tubes. The samples were heated for 5 minutes at 100°C using a Techne Dri-Block DB-3 and centrifuged using a Biofuge bench-top centrifuge at 3000g for 1 minute to precipitate cell debris.

For determination of granulosa cell protein concentration (mg protein/ml), a standard curve was generated from a 2mg/ml stock solution of BSA. To generate the standard curve, the following volumes of stocks were used and diluted in distilled H₂O to produce concentrations of 1.8, 1.5, 1.2, 0.9, 0.6, 0.3, 0.1, and 0mg/ml. Volumes of 10 μ l of each standard were aliquoted into duplicate wells in a 96-well cell culture plate. Into each standard well, a further volume of 10 μ l lysis buffer containing the protease inhibitor cocktail was added (to eliminate any variation caused by the

samples being in lysis buffer containing protease inhibitor cocktail). The samples were diluted 1/2 (v/v) in dH₂O to a total volume of 20µl in duplicate. A reference blank was generated using 10µl lysis buffer containing protease inhibitor cocktail, and 10µl distilled H₂O.

A total volume of 10ml was prepared using the Bicinchoninic acid reagents, A and B at a ratio of 50:1 (v/v) A:B as per the manufacturers' guidelines (Pierce, UK). The reagents were vortexed, and a volume of 200µl was added to each well. The samples were incubated at 37°C for 30 minutes, and the optical density determined using a Dynex Technologies micro-plate reader at a wavelength of 562nm. The Revelation 3.0 software package was used to determine the unknown sample concentrations from the standard curve generated.

Once protein concentrations were determined, 1µl of 1% (w/v) bromophenol blue, and 5µl β-mercaptoethanol were added to each sample. Samples were heated to 100°C for 5 minutes using a Techne Dri-Block DB-3 and centrifuged using a Biofuge 13 bench-top centrifuge (Heraeus Sepatech) at 10,000g for 1 minute to re-precipitate cells.

2.8 SDS-PAGE

SDS-PAGE was used for resolution of 11βHSD1 protein from granulosa cells and rat kidney homogenate samples according to their molecular weights. A BioRad Protean IIxi Gel electrophoresis system was used with reducing polyacrylamide Tris-glycine gels composed of a 4% (w/v) acrylamide stacking gel, and a 10% (w/v)

acrylamide resolving gel (for polyacrylamide gel composition see Appendices I- J and K). A running buffer system of Tris-Gly-SDS was used and the protein samples loaded into the stacking gel at a final concentration of 30µg protein/60µl loading buffer. Molecular weight markers were run alongside each protein sample. Electrophoresis was carried out at a current of 15mA per gel overnight until the gel front reached the bottom of the plates.

2.9 Western Blot

2.9.1 Preparation for transfer of proteins from SDS-PAGE gel to polyvinylidene difluoride (PVDF) membrane

The PVDF membrane and 2 pieces of filter paper were cut to the appropriate size to match the size of the SDS-PAGE gel. To make the membrane receptive to protein transfer, the membrane was saturated in methanol, and transferred to distilled H₂O for 10 minutes. Following this, the PVDF membrane was transferred to Western blotting transfer buffer for 15 minutes. The SDS-PAGE gel was incubated in transfer buffer for 15 minutes. For the last 5 minutes of the incubation, the filter paper was incubated in transfer buffer. The filter paper, membrane, gel and filter paper were arranged on a Transfer-blot SD semi-dry transfer cell (Biorad) in that order. The blot was transferred for 1 hour 45 minutes at 25V, with a current of 0.8mA per cm² of gel.

2.9.2 Blocking of the membrane

Following the protein transfer to PVDF membrane, the gel and filter paper were discarded and the membrane incubated in a blocking solution of 10% (w/v) BSA, 0.001% (w/v) sodium azide in Tris-buffered saline Tween (TBST) for 2-4 hours.

2.9.3 11 β HSD1 primary antibody probing

The membrane was removed from the blocking solution. Following this the membrane was incubated overnight in a 1/100 dilution of sheep anti-human 11 β HSD1 monoclonal antibody (in 10% (w/v) BSA, 0.001% (w/v) sodium azide in TBST) at maximum agitation on a Rotomax 120 shaker (Heidolph). Following this, the membrane was removed from primary antibody and washed for 10 minutes in 150ml TBST at maximum agitation on the Rotamax 120. The TBST washing buffer was discarded and this wash step was repeated a total of 6 times.

2.9.4 Secondary antibody probing

Following the wash steps, the membrane was incubated in a 1/10,000 dilution of donkey anti-sheep IgG (diluted in TBST containing 0.2% (w/v) BSA) conjugated to horseradish peroxidase for 1 hour, at maximum agitation on a Rotamax 120 shaker. After 1 hour, the blot was removed from secondary antibody and washed for 10 minutes in 150ml TBST at maximum agitation. This wash step was repeated a total of 8 times.

2.9.5 Developing of the blot

To visualise the binding of the secondary antibody conjugated to horseradish peroxidase, the membrane was incubated in equal quantities of ECL reagents 1 and 2 for 1 minute as per the manufacturers' guidelines (Amersham Biosciences, UK). The ECL reagents were aspirated from the membrane, discarded and the PVDF membrane was wrapped in saran wrap. The PVDF membrane was placed into a film cassette and protein bands visualised through exposure onto Hyperfilm ECL[®] for 1, 3 or 5 minutes. The film was then developed using the ECL detection method as per the manufacturers' guidelines (Amersham Biosciences, UK).

2.9.6 Stripping the membrane of bound antibody

To re-probe the membrane for GAPDH expression, bound primary antibody was stripped from the membrane. To achieve this, the membrane was incubated in 100ml of stripping buffer (see Appendix I L) at 50°C for 20 minutes in a water-bath (adapted from the manufacturers' guidelines, Amersham Biosciences). Following incubation, the blot was removed and the stripping buffer discarded. The blot was transferred to wash in 150ml TBST at maximum agitation on a Rotamax 120. This wash step was repeated 4 times in preparation for re-probing of the membrane for GAPDH.

2.9.7 GAPDH re-probing

GAPDH was probed for using the same protocol that was used to determine 11 β HSD1 expression but with different antibodies (see section 2.9.3 to 2.9.5). The

primary antibody was a murine antibody raised against the human sequence of GAPDH. The secondary antibody was rabbit anti-mouse IgG used at a dilution of 1/5,000 in TBST containing 0.2% (w/v) BSA.

2.9.8 Quantification of protein expression

Densitometric analysis was carried out to quantify protein expression using a BioRad Model GS-690 Imaging Densitometer with BioRad Molecular Analyst software, version 1.4. A ratio of 11 β HSD1 expression to GAPDH was used to quantify protein expression within a given lane of a Western blot.

2.10 Statistical analyses

All experimental data are presented as the mean \pm SEM of up to 98 independent experiments, performed using cells from individual patients. Due to differences in hormonal responses, hormonal synthesis and enzyme activities between patients, the results of each experiment have been standardised and presented as a percentage of the basal control. However, all statistical analyses were performed using absolute data and not the internally referenced data.

Statistical evaluations were carried out using GraphPad Prism 3.02 software (San Diego, USA). Since data conformed to Gaussian distributions, parametric tests were used. When comparing two means in isolation, an unpaired *t*-Test was used treatments (with Welch's correction where appropriate). When comparing multiple treatments, or a change across a single variable (e.g., concentration of treatment or with varying time), a one-way analysis of variance (ANOVA) with repeated

measures was performed followed by a Dunnett's multiple comparison as the *post hoc* test.

Significance was assessed in all experiments as a probability value of $p < 0.05$.

Chapter Three: Effect of human follicular fluid on the activity and expression
of 11 β HSD1

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11 β HSD1

3.1 Background

Human follicular fluid (FF) is comprised of many compounds including cortisol [Fateh *et al.*, 1989; Jimena *et al.*, 1992]. Several studies have inversely linked the activity of 11 β HSD in granulosa cells, either measured directly *in vitro*, reflected by the ratio of cortisol:cortisone in FF, with the clinical outcome of *in vitro* fertilisation-embryo transfer (IVF-ET) (see section 1.8).

The mammalian body contains a range of natural 11 β HSD enzyme inhibitors including compounds such as bile acids and sterols (Table 1.1). Moreover, studies conducted by Morris *et al.* [1992] first isolated an endogenous inhibitor of 11 β HSD from human urine. This factor was termed ‘glycyrrhetic acid like factor’ (GALF) due to the fact that, like glycyrrhetic acid, GALF was able to inhibit both 11 β HSD and 5 α -reductase activities. The levels of GALF are higher in women than men, and higher in pregnant women than non-pregnant women [Morris *et al.*, 1992].

Inspired by this work, we have speculated that GALF, or related inhibitors of 11 β HSD, may be produced by the ovary and/or placenta. Initial studies conducted by D.P. Norgate suggested that such factors may indeed exist in FF aspirated from women undergoing IVF-ET.

Little is known about the mechanisms by which the modulatory compounds in human FF regulate 11 β HSD activities, and whether the effect is mediated through a change in enzyme expression. Moreover, what the identity of the compounds within human FF are. Therefore the aims of this chapter were:

- (i) To isolate those compounds contained in the hydrophilic and hydrophobic fractions of human FF that acutely modulate 11 β HSD1 activity and characterise their effects on NADP(H)- and NAD⁺ - dependent 11 β HSD activities.
- (ii) To investigate possible candidates for the lipid inhibitors of 11 β HSD1 activity contained in human FF.
- (iii) To investigate the effects of the enzyme modulators in the active fractions of human FF on the expression of 11 β HSD1 in human granulosa-lutein cells.

3.2 Experimental protocol

For all experiments, fractions of human FF, aspirated from the ovaries of women undergoing oocyte retrieval for IVF-ET, were separated by reverse phase C18 column chromatography using a stepwise methanol gradient as described in section 2.6. To assess the effects of specific fractions of human FF on 11 β HSD activities, a rat kidney homogenate assay was used as described in section 2.7. Enzyme activities were assessed over 1 hour in the presence and absence of specific fractions of human FF. Control samples were incubated with 20% (v/v) methanol and test samples were incubated with human FF, or specific fractions thereof, each at a final dilution of

10% by volume. Hence, in all samples, the final methanol concentration was $\leq 2\%$ by volume. At the end of each experiment, the steroids were chloroform extracted, resolved by TLC and cortisol-cortisone inter-conversion quantified using a radiochromatogramme scanner (see section 2.4).

To assess the effects of human FF fractions on 11β HSD1 expression, hGL cells were isolated and seeded into 6 well plates at a density of 1×10^6 cells/2ml of medium per well. Cells were cultured for 2 days in serum-supplemented medium to allow for luteinisation and for cell attachment to the plates (see section 2.2). Following this, cells were transferred to serum-free media, and individual wells were treated with control (20% v/v methanol), human FF that had not been subjected to column chromatography, compounds eluted specifically in the hydrophilic fractions of human FF at 0% (v/v) methanol or the hydrophobic fraction of human FF eluted at 80% (v/v) methanol respectively. All fractions (both the control and experimental fractions of human FF) were diluted to a final concentration of 10% (v/v) and hGL cells were treated for 24 hours. Effects on 11β HSD1 protein expression were determined by SDS-PAGE and Western blot analysis with internal standardisation to GAPDH expression (see sections 2.8, 2.9 and 2.10).

To investigate whether $\text{PGF}_{2\alpha}$, PGE_2 and specific steroids could be the compounds contained in any fractions of human FF found to modulate the activity of 11β HSD, aliquots of human FF were spiked with each tritiated radiochemical as described in section 2.6. To assess the retention of each compound on the C18 column, each compound was eluted by stepwise methanol gradient fractionation using reverse

phase C18 column chromatography (see section 2.6). To confirm the concentration of methanol required to elute each radiochemical, 100µl of each fraction was placed into a scintillation vial, along with 2ml Ultima-gold scintillant. The radioactivity of each fraction was assessed using a liquid scintillation counter and the data standardised relative to the total amount of radioactivity recovered from the C18 column for that specific radiochemical (to 100% as the total compound concentration per column).

It should be noted that the studies of the effects of human FF on enzyme activities and the candidate compound elution profiles in this chapter were conducted in collaboration with L.M. Thurston and D.P. Norgate and have been published in *Reproduction*, (Thurston *et al.*, 2002).

3.3 Results

3.3.1 Effects of human FF on NADP(H)-dependent 11βHSD activities

The component fractions of human FF were resolved using reverse phase C18 column chromatography and the effects of each component fraction of human FF on NADP(H)-dependent 11βHSD activities were tested. Hydrophilic compounds eluted by 0% (v/v) and 10% (v/v) methanol significantly increased NADP⁺-dependent cortisol oxidation to $155.0 \pm 4.5\%$ and $128.5 \pm 3.3\%$ of control enzyme activities respectively ($p < 0.01$, Figure 3.1). In contrast, hydrophobic compounds eluted at 75% (v/v), 80% (v/v) and 85% (v/v) methanol inhibited NADP⁺-dependent cortisol oxidation. Maximum enzyme inhibition was achieved by those hydrophobic fractions of human FF eluted at 80% (v/v) methanol. This fraction of human FF

inhibited cortisol oxidation by $63.1 \pm 3.7\%$ ($p < 0.01$) in comparison to control. The effects of each component fraction of human FF on NADPH-dependent cortisone reduction mirrored those on cortisol oxidation. Hydrophilic compounds eluted by 0% (v/v) methanol increased cortisone reduction to $185.7 \pm 2.2\%$ of control ($p < 0.01$), whereas, hydrophobic compounds eluted at 75% (v/v), 80% (v/v) and 85% (v/v) methanol inhibited NADPH-dependent cortisone reduction by up to $73.8 \pm 1.5\%$ of control enzyme activity ($p < 0.01$, Figure 3.2).

To assess whether the active compounds contained in human FF were lipids, 6 randomised human FF samples were subjected to charcoal-stripping before being applied to a C18 cartridge for methanol resolution. Charcoal-stripping of the human FF had no effect on compounds eluted at 0% (v/v) methanol, which continued to stimulate NADP^+ -dependent cortisol oxidation. However, charcoal-stripping of human FF effectively removed the hydrophobic inhibitors of NADP(H) -dependent $11\beta\text{HSD}$ activities eluted at 75 % (v/v) to 85% (v/v) methanol (Figure 3.3).

3.3.2 Effects of human FF on NAD^+ -dependent cortisol oxidation

Fractions resolved from human FF using reverse phase C18 column chromatography had no affect on NAD^+ -dependent cortisol oxidation when assessed at a final dilution of 10% by volume ($p > 0.05$, Figure 3.4).

3.3.3. Elution profiles of ^3H lipids from C18 cartridges

Using the same reverse phase C18 chromatography profile, cortisol and cortisone eluted at 45% (v/v) and 50% (v/v) methanol, respectively, while oestradiol, testosterone, progesterone and pregnenolone eluted over the range of 60% (v/v) to 75% (v/v) methanol. Cholesterol was eluted in the 100% (v/v) methanol fraction, whereas PGE_2 and $\text{PGF}_{2\alpha}$ eluted in the range of 20-40% (v/v) methanol (Figure 3.5).

3.3.4 Effect of human FF fractions on $11\beta\text{HSD1}$ expression in hGL cells

To assess the effect of human FF fractions on $11\beta\text{HSD1}$ protein expression in hGL cells, 9 human FF samples were chosen from the cohort of human FF assessed in section 3.3.1 and fractionated using a methanol gradient. Human FF that had not been subjected to C18 column chromatography (whole human FF) and the active fractions of human FF eluted at 0% and 80% (v/v) methanol had no effect on $11\beta\text{HSD1}$ protein expression in hGL cells ($p > 0.05$, Figure 3.6).

3.4 Discussion

Previous work conducted in the laboratory by D.P. Norgate had indicated that human FF might contain hydrophilic and hydrophobic compounds that could modulate the inter-conversion of cortisol and cortisone by $11\beta\text{HSD}$. Work conducted in collaboration with Thurston *et al.* [2002] has revealed that the modulators in human FF can be resolved by reverse phase C18 column chromatography using stepwise

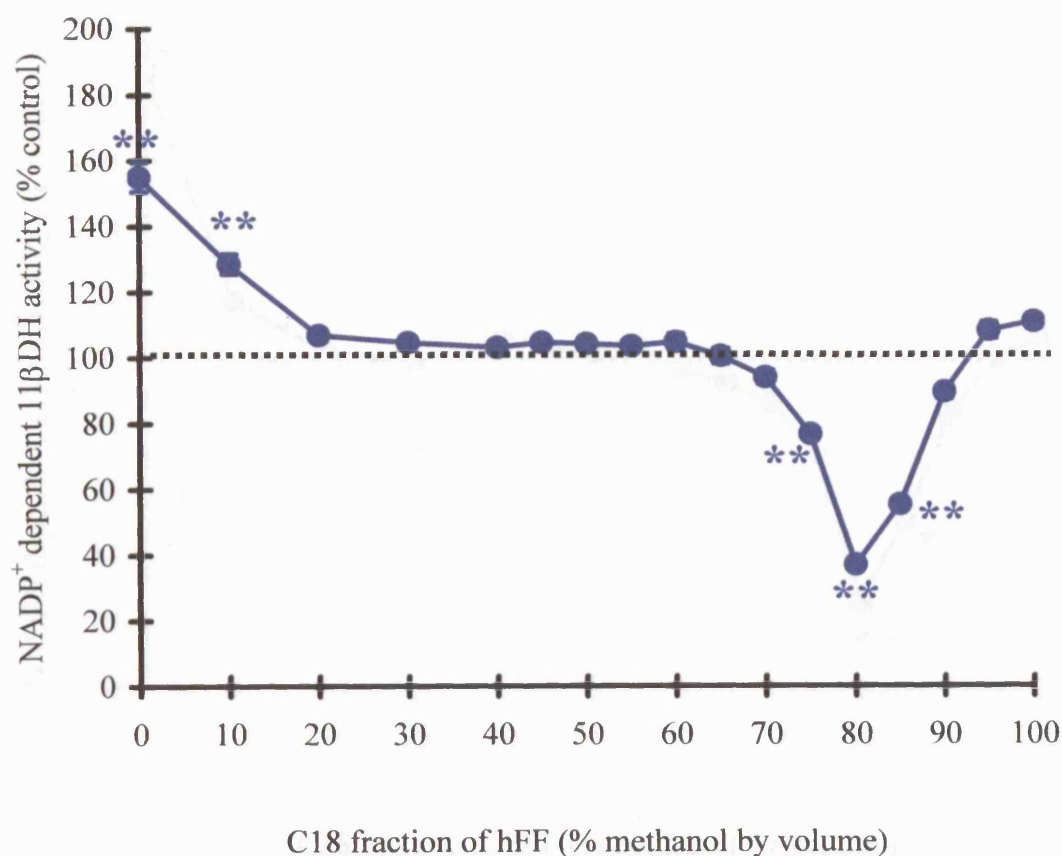


Figure 3.1: Effects of compounds in human FF eluted by reverse-phase C18 chromatography using a stepwise methanol gradient fractionation on NADP⁺-dependent oxidation of 100nM cortisol over 1 hour in a rat kidney homogenate bioassay. Data represent the mean \pm SEM for 98 samples of human FF, (** $p < 0.01$ versus no human FF fraction). Data subjected to one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

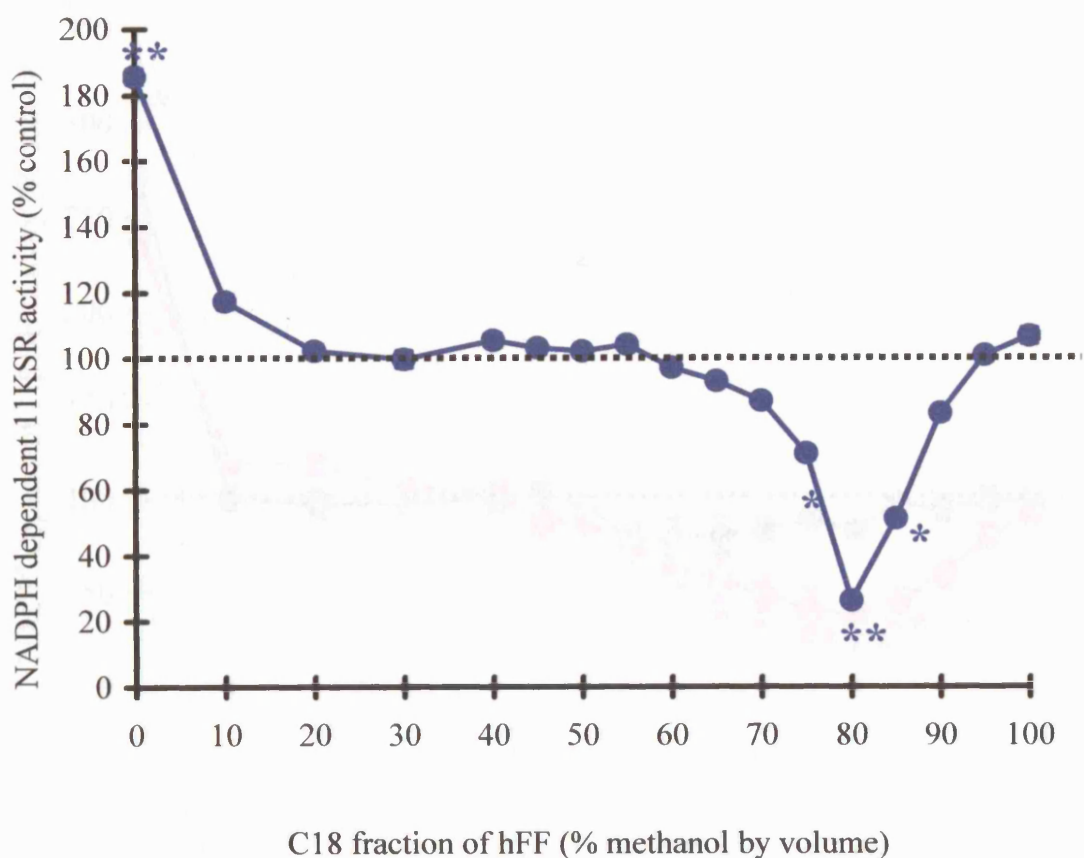


Figure 3.2: Effect of compounds in human FF eluted by reverse-phase C18 chromatography and stepwise methanol gradient fractionation on NADPH-dependent reduction of 100nM cortisone over 1 hour in a rat kidney homogenate bioassay. Data represent the mean \pm SEM for 6 samples of human FF, (** $p < 0.01$, versus no human FF). Data subjected to one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

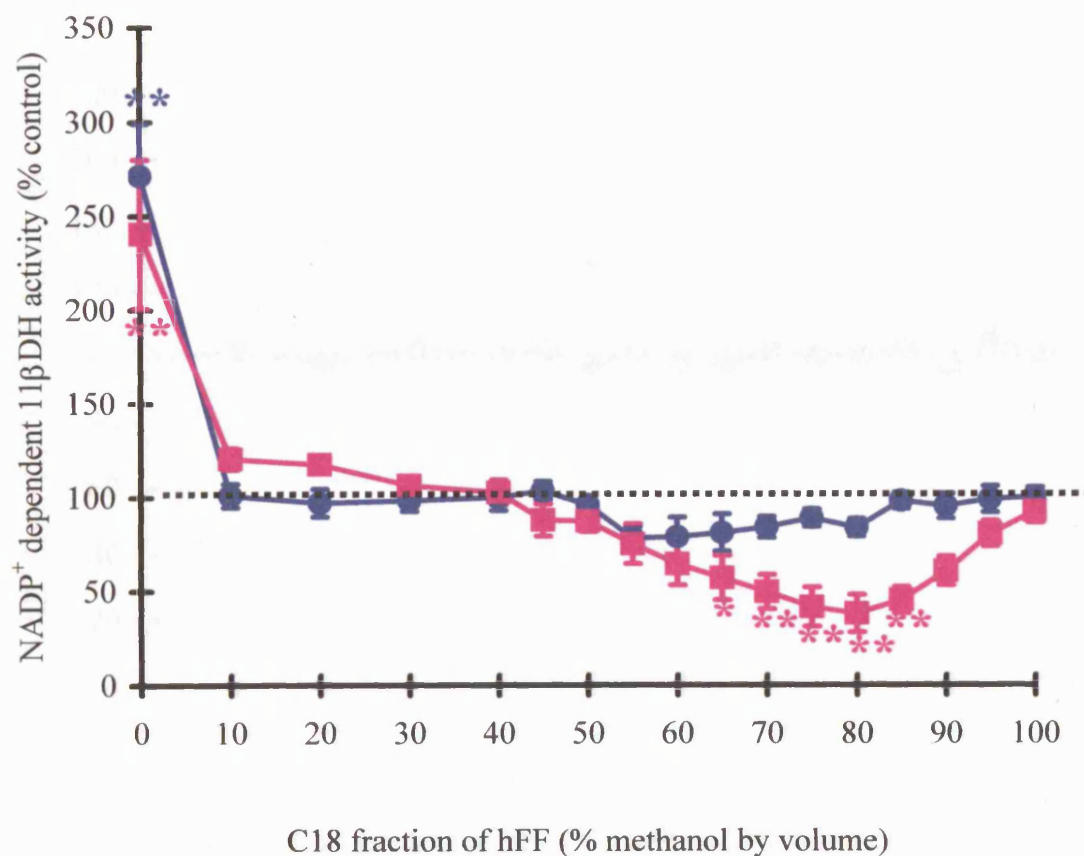


Figure 3.3: Effect of compounds in human FF before (pink line) and after (blue line) incubation with dextran-coated charcoal. Fractions were eluted by reverse-phase C18 chromatography using a stepwise methanol gradient fractionation on NADP⁺-dependent oxidation of 100nM cortisol over 1 hour. Data represent the mean \pm SEM for 6 samples of human FF, (* p <0.05, ** p <0.01 versus no human FF). Data subjected to one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

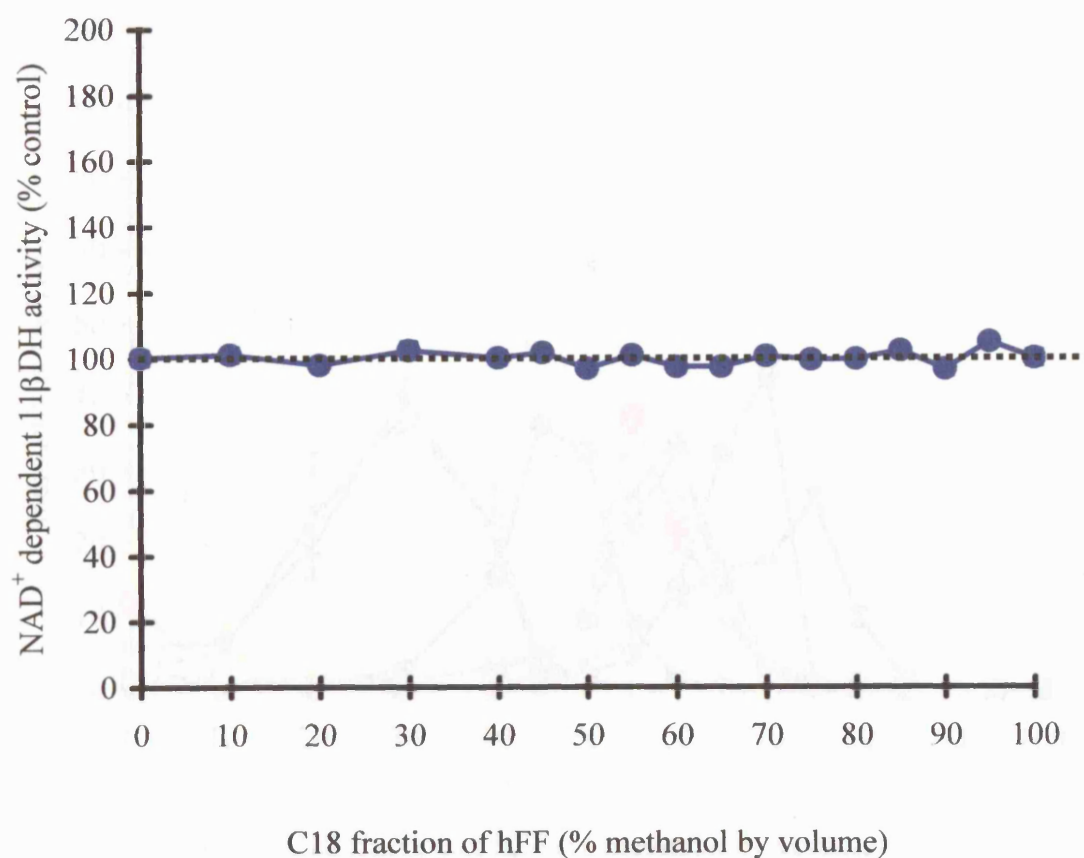


Figure 3.4: Effect of compounds in human FF eluted by reverse-phase C18 chromatography and stepwise methanol gradient fractionation on NAD⁺-dependent oxidation of 100nM cortisol over 1 hour in a rat kidney homogenate bioassay. Data represent the mean \pm SEM for 58 samples of human FF, ($p > 0.05$ versus human FF). Data subjected to one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

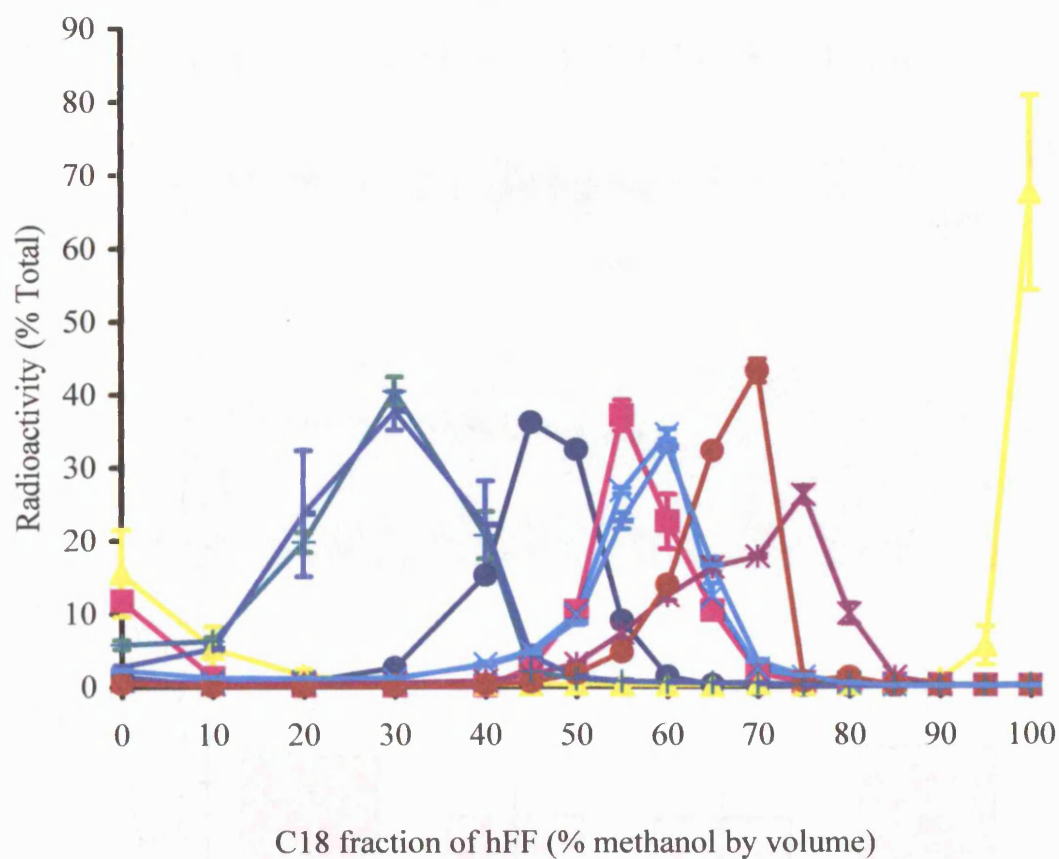


Figure 3.5: Elution profiles of exogenously added prostaglandins and steroids in human FF eluted by reverse-phase C18 chromatography and stepwise methanol gradient fractionation. Yellow triangle=cholesterol, purple cross=pregnenolone, Brown circle=progesterone, Blue line=testosterone, Blue cross=oestradiol, Pink square=cortisone, Blue circle=cortisol, Green cross=PGE₂, Light Blue line=PGF₂α. Data represent the mean \pm SEM for 3 samples of human FF.

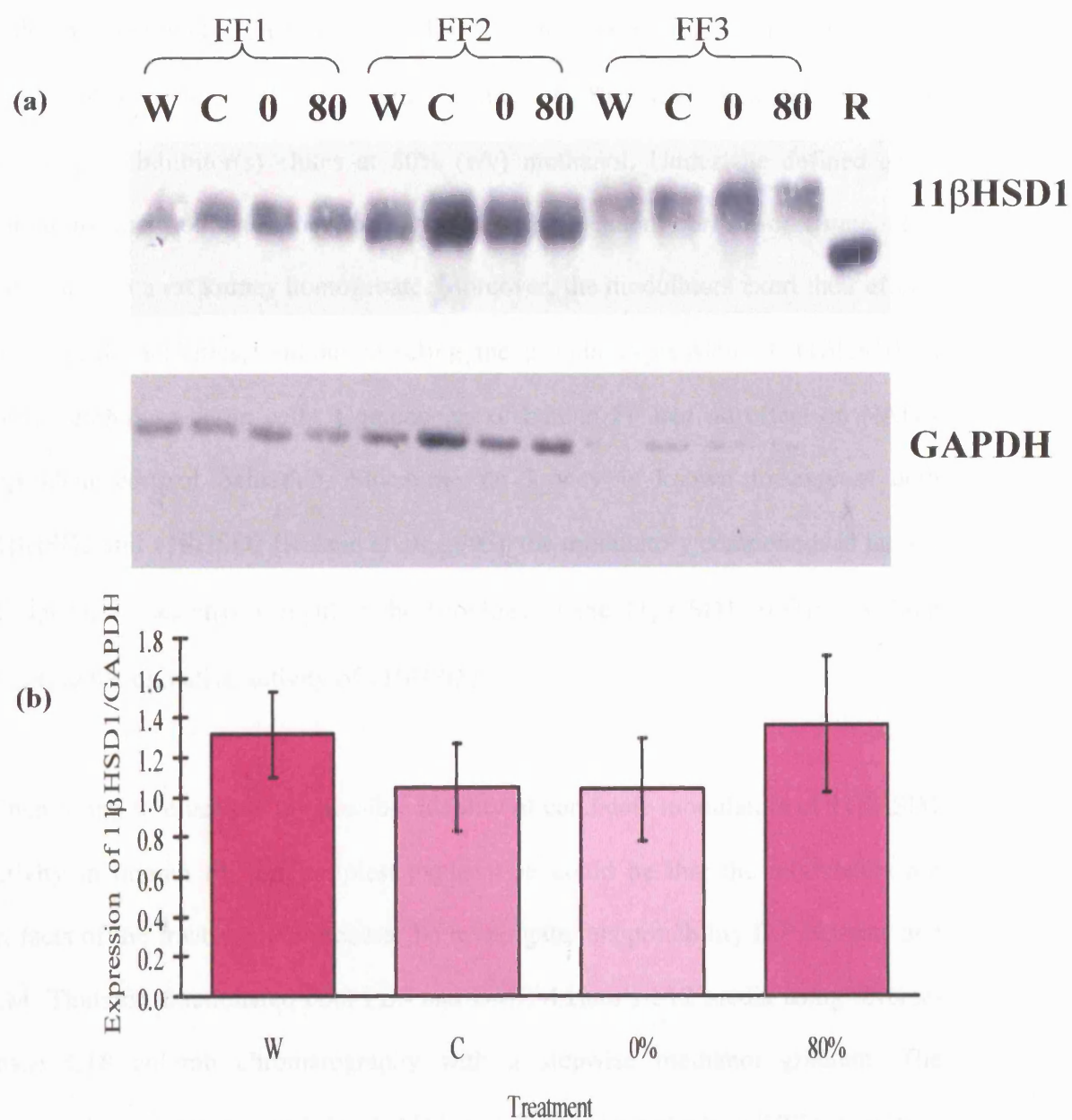


Figure 3.6: (a) Representative Western blot of effect of whole non-fractionated hFF (W), and those fractions of human FF eluted at 0% (v/v) and 80% (v/v) methanol on expression of 11HSD1 (and GAPDH) in hGL cells. W= whole non-fractionated hFF, C=control, 0= compounds eluted with 0% (v/v) methanol, 80= compounds in hFF eluted with 80% (v/v) methanol, R=positive control of rat kidney **(b)** Densitometric representation of effect of hFF and specific fractions thereof on 11βHSD1 protein expression standardised to GAPDH. Data represent the mean ± SEM for 9 independent samples (n=9). Means compared at each condition using paired T-test.

methanol gradient fractionation. Using this technique, the hydrophilic stimulus/stimuli to 11 β HSD activities elute at 0% (v/v) methanol, and the hydrophobic inhibitor(s) elutes at 80% (v/v) methanol. Under the defined assay conditions, the modulators only affect NADP(H)-dependent cortisol-cortisone inter-conversion in a rat kidney homogenate. Moreover, the modulators exert their effects on 11 β HSD activities, without affecting the protein expression of 11 β HSD1 in human granulosa-lutein cells. Components of human FF had no effect on NAD⁺-dependent cortisol oxidation. Since the rat kidney is known to express both 11 β HSD1 and 11 β HSD2 [Roland *et al.*, 1995], the modulatory compounds in human FF appear to selectively regulate the activities of the 11 β HSD1 isoform, without affecting the oxidative activity of 11 β HSD2.

When trying to elucidate the possible identity of candidate modulators of 11 β HSD1 activity in human FF, the simplest explanation could be that the modulators are artifacts of the fractionation process. To investigate this possibility D.P Norgate and L.M. Thurston fractionated both PBS and DMEM:Ham's F12 media using reverse-phase C18 column chromatography with a stepwise methanol gradient. The generated fractions assayed for NADP and NAD dependent-11 β HSD activities. Neither PBS or DMEM:Ham's F12 had any modulatory effect on 11 β HSD activities. Thus the modulatory compounds of 11 β HSD1 activity resolved by reverse-phase C18 column fractionation do appear to originate from human FF.

Although outside the remit of the work reported in this Chapter, studies to investigate whether the modulators of 11 β HSD1 activity in human FF were GALF

have been conducted [Thurston *et al.*, 2002]. Urine samples from the same cohort of patients from which the FF was obtained was taken 2 hours prior to FF collection, and subjected to reverse phase C18 column fractionation using a stepwise methanol gradient. No correlation was seen between the fractionation profile of the modulators in human FF and the modulators in human urine, or GALF. Therefore the modulators in human FF were not those previously identified by Morris *et al.*, [1992].

Possible identities for the hydrophilic stimuli of 11 β HSD1 include proteins and water-soluble metabolites. Approximately 97% of the total protein content of human FF is eluted from a C18 column in the loading eluent of whole human FF and the 0% (v/v) methanol fraction of human FF [Thurston *et al.*, 2002]. A possible water-soluble metabolite that could stimulate the activities of 11 β HSD1 is glucose-6-phosphate. Recent publications have linked the activities of 11 β HSD1 with the metabolism of glucose-6-phosphate/fructose-6-phosphate by hexose-6-phosphate dehydrogenase (H6PDH) [Banhegyi *et al.*, 2004]. Moreover, it has been speculated that functional co-operativity exists between the two enzymes both localised to the lumen of the ER, suggesting a link between the regulation of glucose metabolism, and the functionality of 11 β HSD1 [Atanasov *et al.*, 2004].

Charcoal-stripping to remove lipids from human FF has shown that the hydrophobic enzyme inhibitors of 11 β HSD in FF are lipids. The addition of exogenous tritiated prostaglandins, steroids and cholesterol to human FF has eliminated these compounds as candidates contained in the hydrophobic fraction of human FF eluted

at 80% (v/v) methanol, since these candidate lipids eluted at 30-40% (v/v), 50-75% (v/v) and 100% (v/v) methanol respectively.

Recent studies have shown that 7-oxysterols can act as substrates for 11 β HSD1 [Hult *et al.*, 2004]. Studies have shown that human, rat and mouse 11 β HSD1 can inter-convert 7-ketocholesterol and 7 β -hydroxycholesterol with similar k_{cat} values as observed with glucocorticoids [Hult *et al.*, 2004; Schweizer *et al.*, 2004]. These studies suggest that 7-oxysterols could be modulatory components of human FF that could compete for the active site of 11 β HSD1, and competitively inhibit cortisol-cortisone inter-conversion. The structural properties of the oxysterols would render them less hydrophobic than cholesterol, but more hydrophobic than known steroids. Hence, they might be expected to elute from a C18 column at 80% (v/v) methanol.

Another possible candidate for the intrafollicular 11 β HSD inhibitor eluted with 80% (v/v) methanol could be meiosis activating sterol (MAS). This sterol has been isolated from pre-ovulatory human FF [Byskov *et al.*, 1995] and the concentration of MAS has been linked to the ability of the human oocyte to fertilise and cleave [Byskov *et al.*, 1998]. Other candidates for the hydrophobic inhibitors of 11 β HSD1 could be bile acids and their derivatives. Bile acids are also derived from cholesterol, are comprised of 24 carbons and are hydroxylated. These structural properties render bile acids (like the oxysterols) less hydrophobic than cholesterol, but more hydrophobic than steroids. Bile acids such as chenodeoxycholic acid, have also been shown to inhibit the activity of 11 β HSD1 [Perschel *et al.*, 1991; Buhler *et al.*, 1994; Morris and Souness., 1996; Diederich *et al.*, 2000; Morris *et al.*, 2004]. However,

bile acids also inhibit NAD^+ -dependent cortisol oxidation by $11\beta\text{HSD2}$ such that they are unlikely to be the main inhibitory compound that specifically inhibits $11\beta\text{HSD1}$ activities in human FF.

Chronic exposure of hGL cells to whole human FF (not subjected to C18 cartridge chromatography) and human FF fractions eluted at 0% (v/v) or 80% (v/v) methanol had no significant effect on $11\beta\text{HSD1}$ protein expression. These data suggest that the components of human FF affect $11\beta\text{HSD1}$ activities without affecting $11\beta\text{HSD1}$ enzyme expression, instead acting via a post-translational mechanism. Since $11\beta\text{HSD1}$ binds cortisol and cortisone in a hydrophobic binding cleft [Tsigelny and Baker, 1995b], it is possible that lipids from human FF could inhibit $11\beta\text{HSD1}$ activities by competing with cortisol and cortisone for binding to the active site of the enzyme. Published hydropathy plots also indicate that the human $11\beta\text{HSD1}$ protein contains hydrophobic regions outside the active site, which could bind hydrophobic allosteric regulators of $11\beta\text{HSD1}$ (The structural and functional significance of the hydrophobic regions in human $11\beta\text{HSD1}$ are investigated by the studies reported in Chapter 7).

Limitations to this study are that (i) concentration-dependent effects and (ii) kinetic analysis of the stimulatory and inhibitory compounds were not determined. If additional research time were permitted the concentration dependent effects of human FF could be tested in the rat kidney homogenate radiometric conversion assay to determine effects of each eluted fraction on $11\beta\text{HSD}$ enzyme activities. A concentration range of 0% (v/v), 2% (v/v), 5% (v/v), 10% (v/v) and 20% (v/v) would

be used for each reverse phase C18 column eluted fraction of human FF. A maximum concentration of 20% (v/v) of each fraction would be used in this assay to avoid methanol toxicity.

Kinetic analysis of the stimulatory and inhibitory fractions of human FF could be assessed using the rat kidney homogenate radiometric conversion assay. However, due to the crude nature of the purification method for isolating these modulatory compounds, it is possible that each fraction is comprised of multiple modulatory factors. Therefore, in order to determine the kinetic properties of a specific modulator within these fractions, further purification of the components of each FF fraction would first be needed. Techniques that could be employed to do this are gas-chromatography followed by mass spectrometry. Furthermore, following purification the individual compounds from each modulatory fraction would need to be isolated in sufficient quantities before kinetic analysis could be carried out.

The human FF used in these studies was obtained from women undergoing controlled ovarian hyperstimulation for IVF-ET. Therefore the presence of the enzyme modulators could be the result of pituitary down-regulation and ovarian hyperstimulation, as opposed to a natural phenomenon in the human ovary. In subsequent studies conducted by Thurston *et al.* [2003c], it has been established that bovine and porcine FF also contain compounds that can stimulate and inhibit the NADP(H)-dependent activities of 11 β HSD1. Moreover, these compounds isolated from bovine and porcine FF have biophysical properties comparable to the enzyme modulators isolated from human FF. Since these modulatory compounds in bovine and porcine FF were isolated from animals that have not been subjected to any

endocrine therapy, it is likely that the modulators of 11 β HSD1 activities in human FF are a natural phenomenon and not an artefact of IVF-ET.

In conclusion, endogenous modulators in human FF regulate both the oxidative and reductive activities of 11 β HSD1, but do not affect enzyme expression, suggesting possible post-translation mechanisms for the regulation of 11 β HSD1 by compounds present in the human ovary. Possible candidates for these stimulatory and inhibitory modulators are likely to be hydrophilic proteins/metabolites and hydrophobic sterols respectively. Although steroids and prostaglandins were not found to be in the active fractions of human FF that regulate 11 β HSD1 activity, these lipids have characterised functions in the regulation and control of the ovarian cycle. Therefore the next 3 chapters will address the modulatory roles for progesterone and prostaglandins in the regulation of 11 β HSD1 activities and expression in hGL cells.

**Chapter Four: The autocrine/paracrine effects of progesterone on the activities
and expression of 11 β HSD1 in human granulosa-lutein cells**

Chapter 4-The autocrine/paracrine effects of progesterone on the activities and expression of 11 β HSD1 in human granulosa-lutein cells

4.1 Introduction

The expression of 11 β HSD isoforms in the ovary has been well documented. Rat [Tetsuka *et al.*, 1999a], bovine [Tetsuka *et al.*, 2003] and human [Michael *et al* 1997; Tetsuka *et al.*,1997; Ricketts *et al.*, 1998] ovaries have all been shown to express 11 β HSD isoforms differentially dependent on the stage of the ovarian cycle. In the follicular phase, granulosa cells co-express mRNA encoding 11 β HSD2 and mineralocorticoid receptors [Tetsuka *et al.*, 1999a]. In contrast, following the administration of an ovulatory dose of LH/hCG, 11 β HSD2 mRNA is down-regulated and 11 β HSD1 mRNA expression is up-regulated (see section 1.8.4).

There are numerous endocrine and paracrine regulators of 11 β HSD (see Table 1.1 for summary). In the ovary, there are many secretory hormones that regulate the ovarian cycle. Progesterone is the major secretory product of the corpus luteum, synthesised by the luteinising granulosa and theca cells. In other cell types, including the kidney, progesterone and its metabolites have been shown to regulate the activity of 11 β HSD [Quinkler *et al.*, 1999; Burton and Waddell 2002; Quinkler *et al.*, 2003]. However, the potential autocrine/paracrine effects of progesterone on 11 β HSD activity and expression in luteinising human granulosa cells have yet to be elucidated. Therefore the aims of this study were:

- (i) To determine whether aminoglutethimide (AG) can inhibit progesterone synthesis in human granulosa-lutein (hGL) cells.

- (ii) To investigate the effects of inhibition of progesterone synthesis on 11 β HSD activities in human granulosa lutein-cells.
- (iii) To determine if any effect observed as a result of progesterone inhibition on 11 β HSD enzyme activity is dependent on changes in 11 β HSD1 protein expression in human granulosa lutein-cells.

4.2 Experimental Design

To investigate the effects of AG on 11 β HSD1 activities and expression in human granulosa lutein-cells, cells were isolated from follicular aspirates from women undergoing IVF-ET (section 2.2). Cells were seeded at a density of 5×10^4 cells/ml medium in 24 well plates for activity studies and progesterone secretion studies or at 1×10^6 cells/2ml medium in 6 well plates for expression studies. The cells were cultured for 2 days in serum-supplemented medium to allow for luteinisation and attachment to the plates, and transferred to serum-free medium to assess the effect of AG on the activities and expression of 11 β HSD (section 2.2).

To assess acute chronic affects of AG exposure, human granulosa-lutein cells were incubated with increasing concentrations of AG (0 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M, 100 μ M) for 4 and 24 hours respectively. 4 and 24 hour time points were used to assess whether any effects of AG were via signalling mediated events (typically carried out over minutes to hours) or via changes in gene expression, leading to changes in protein expression (typically carried out over hours to days). The vehicle to solubilise AG was dimethyl sulphoxide (DMSO). Each concentration of AG was prepared in a final concentration of 0.1% (v/v) DMSO, and control wells contained

0.1% (v/v) DMSO as vehicle. The acute effects of AG were determined for 4 hours, coinciding with the last 4 hours of the 24 hour treatment incubation.

To verify that AG inhibited progesterone production, parallel cultures were set up and treated as for the enzyme activity studies. Progesterone concentrations were resolved measured in the spent serum-free medium following 4 or 24 hours treatment at each concentration of AG using a radioimmunoassay (section 2.3.1).

11 β HSD activities were assessed over 4 hours in the presence of each concentration of AG using a radiometric conversion assay (section 2.4). In the case of cells exposed to AG for 24 hours, enzyme activities were assessed in the final 4 hour time period (i.e. the same time point as for cells treated acutely with AG). Both 11 β -dehydrogenase (11 β DH) and 11-ketosteroid reductase (11KSR) activities were assessed, steroids extracted and cortisol-cortisone conversion quantified as described in section 2.4.

For the assessment of changes in 11 β HSD1 protein expression, human granulosa-lutein cells were incubated for either 4 or 24 hours with that concentration of AG shown to have the greatest effect on 11 β HSD activities (100 μ M). As for activity studies, the 4 hour time period coincided with the last 4 hours of the 24 hour treatment incubation. Cell proteins were prepared and separated by SDS-PAGE, and 11 β HSD1 protein expression was assessed using Western blot, quantified by densitometric analysis, and standardised to GAPDH protein expression (see sections 2.8 to 2.10).

4.3 Results

4.3.1 Concentration-dependent effects of exposure to AG on progesterone production in human granulosa-lutein cells

In human granulosa-lutein cells acutely treated for 4 hours, AG inhibited progesterone production in a concentration-dependent manner (Figure 4.1). Maximum inhibition of progesterone production was observed in cells treated with 100 μ M AG which inhibited progesterone production by $61.5 \pm 3.4\%$ of control ($p < 0.01$). Incubation of human granulosa-lutein cells with AG for 24 hours also inhibited progesterone production in a concentration-dependent manner, with 100 μ M AG inhibiting progesterone production by up to $95.5 \pm 1.2\%$ of control ($p < 0.01$, Figure 4.1).

4.3.2 Concentration dependent effects of AG on 11 β HSD activities in human granulosa-lutein cells

Acute treatment of human granulosa-lutein cells with AG for 4 hours increased both the 11 β DH and 11KSR activities in a concentration-dependent manner (Figure 4.2). 11 β DH activity was significantly increased to $150.2 \pm 22.2\%$ of control ($p < 0.05$) and 11KSR activity was significantly increased to $228.3 \pm 60.0\%$ of control ($p < 0.05$) by 100 μ M AG.

Chronic exposure of human granulosa-lutein cells to AG for 24 hours revealed a similar trend as with acutely treated cells, increasing both 11 β DH and 11KSR activities (Figure 4.3). Treatment of human granulosa-lutein cells with 100 μ M AG

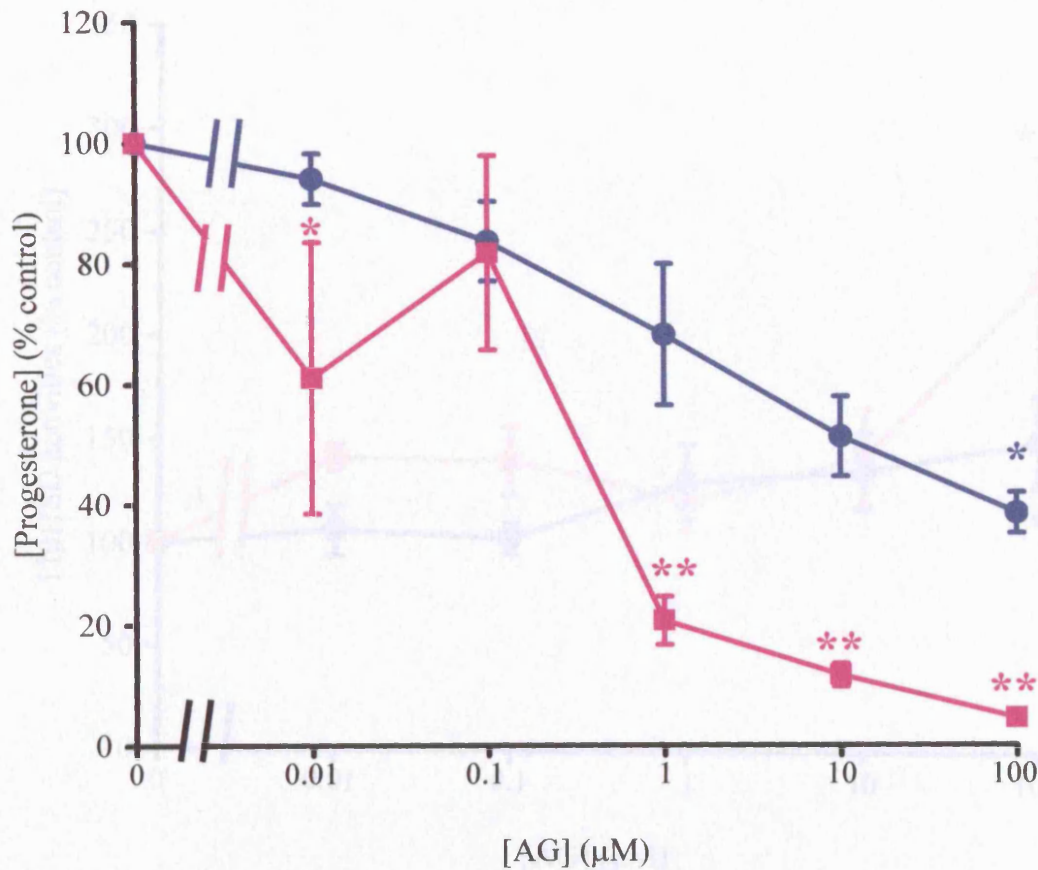


Figure 4.1: Concentration-dependent effects treatment with AG for 4 hours (blue line) or 24 hours (pink line) by human granulosa-lutein cells on day 3 of culture. Both data sets represent the mean \pm SEM for 3 independent experiments, (* $p < 0.05$, ** $p < 0.01$ versus 0 μ M AG). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison for each duration of treatment.

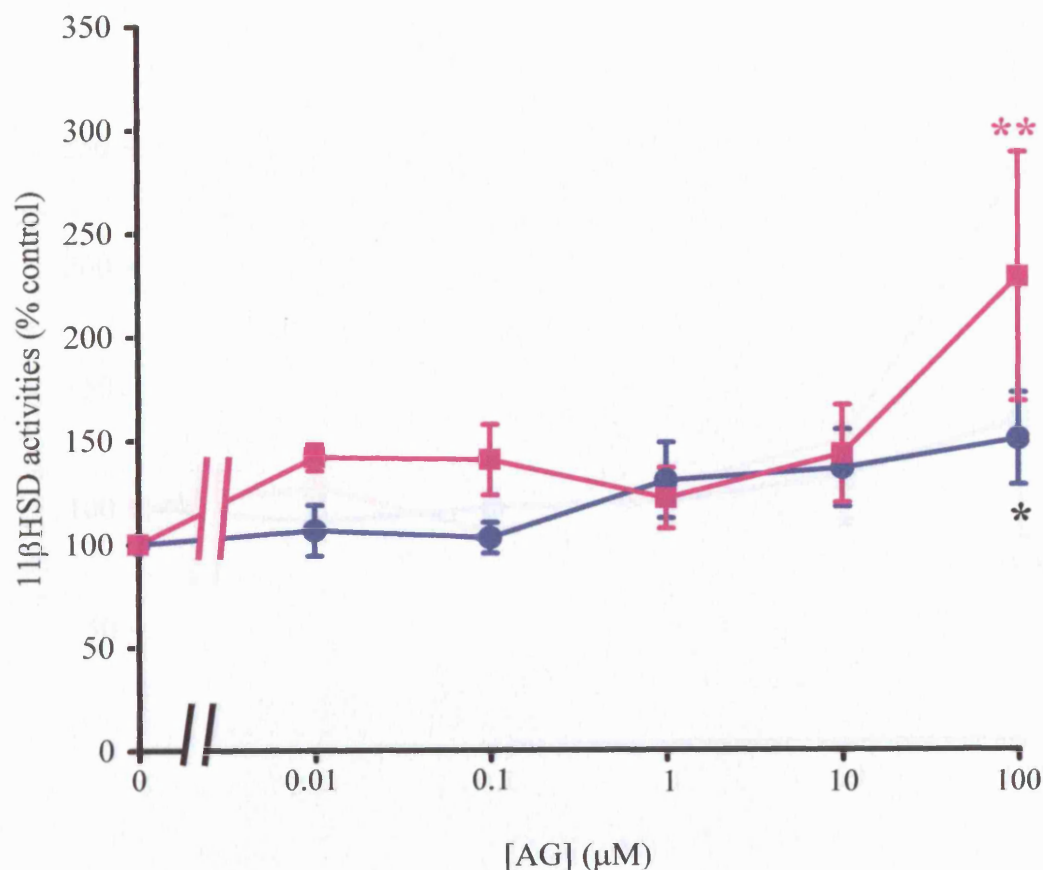


Figure 4.2: Concentration-dependent effects of AG on 11βHSD activities over 4 hours on day 3 of culture in human granulosa-lutein cells. The blue line represents 11βDH activity. All data points represent mean \pm SEM for six independent experiments, (* $p < 0.05$ versus 0μM AG). The pink line represents experiments 11KSR activity. All data points represent the mean \pm SEM for five independent experiments, (** $p < 0.01$ versus 0μM AG). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison for each enzyme.

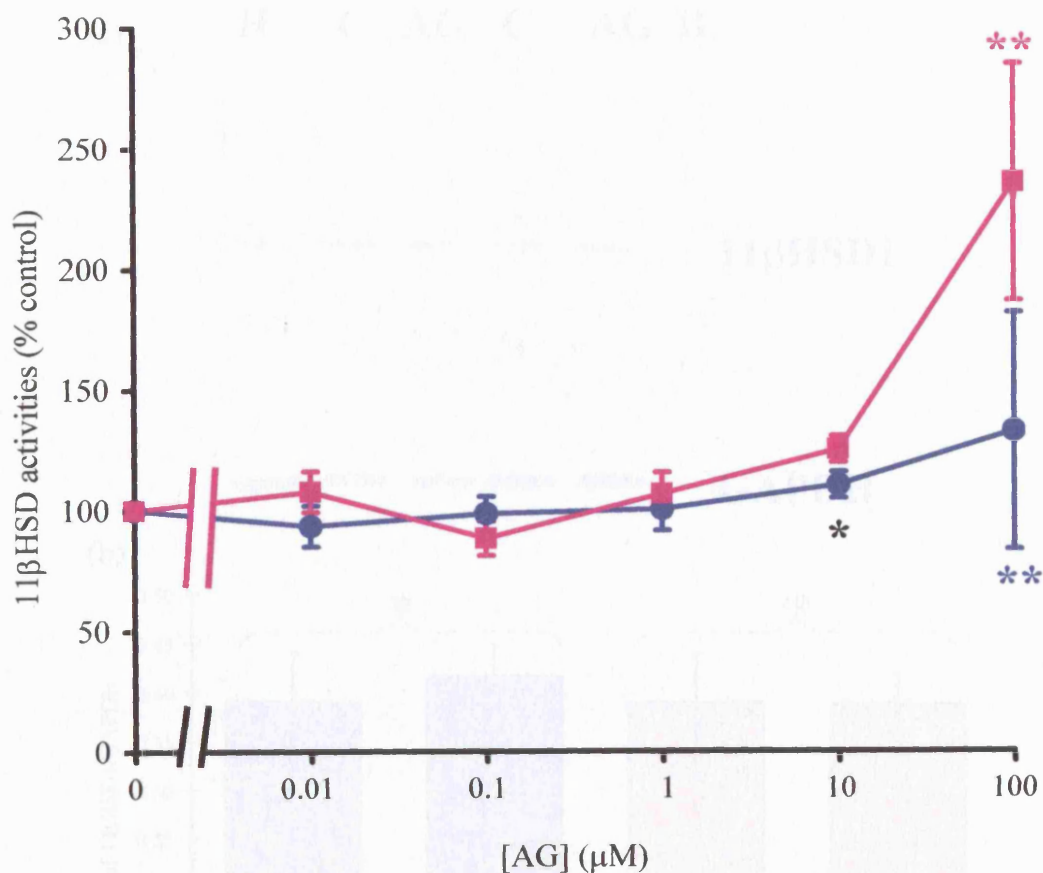


Figure 4.3: Concentration dependent effects of AG on 11βHSD activities over 24 hours on day 3 of culture in human granulosa-lutein cells. The blue line represents 11βDH activity. The pink line represents experiments 11KSR activity. Both data sets represent the mean \pm SEM for 3 independent experiments (** $p < 0.01$ versus 0 μM AG). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison for each enzyme activity.

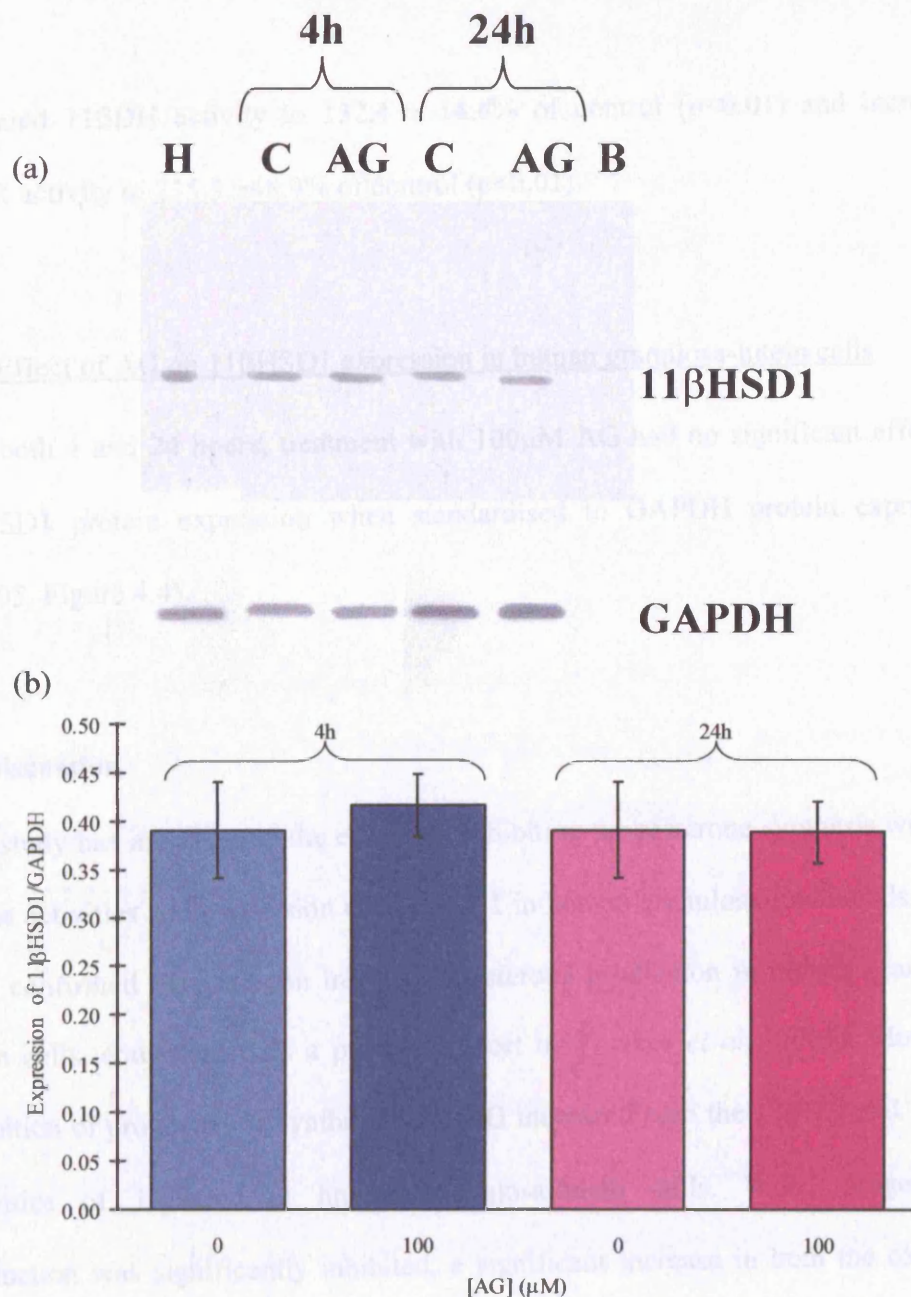


Figure 4.4: Effects of AG on 11HSD1 protein expression (a) Western blot showing effect of AG on 11βHSD1 protein expression relative to GAPDH on day 3 of culture in human granulosa lutein cells. H represents HEK293 cells stably transfected with 11βHSD1 as a positive control, C represents control conditions of just vehicle (0.1% DMSO), AG represents treatment with 100μM AG, and B represents a blank well. (b) Densitometric representation of the effect of AG on 11βHSD1 protein expression relative to GAPDH on day 3 of culture in human granulosa lutein cells The blue bars represent experiments carried out over 4 hours. The pink bars represents experiments carried out over 24 hours. Hatched bars represent control, and solid bars represent hGL cells treated with 100μM AG. All data points represent mean ±SEM for 10 independent experiments $p > 0.05$. Means compared at each time point using unpaired T-test.

stimulated 11 β DH activity to $132.4 \pm 14.6\%$ of control ($p < 0.01$) and increased 11KSR activity to $235.3 \pm 48.9\%$ of control ($p < 0.01$).

4.3.3 Effect of AG on 11 β HSD1 expression in human granulosa-lutein cells

Over both 4 and 24 hours, treatment with 100 μ M AG had no significant effect on 11 β HSD1 protein expression when standardised to GAPDH protein expression ($p > 0.05$, Figure 4.4).

4.4 Discussion

This study has investigated the effects of inhibiting progesterone synthesis with AG on the activities and expression of 11 β HSD1 in human granulosa-lutein cells. It has been confirmed that AG can inhibit progesterone production in human granulosa-lutein cells, consistent with a previous report by Fowkes *et al*, [2001]. Moreover, inhibition of progesterone synthesis with AG increased both the 11 β DH and 11KSR activities of 11 β HSD in human granulosa-lutein cells. When progesterone production was significantly inhibited, a significant increase in both the oxidative and reductive activities of 11 β HSD was observed. Previous studies have highlighted the ability of progesterone and hydroxylated progesterone metabolites to inhibit 11 β HSD activities [Lopez-Bernal, 1980]. Hence the positive effect of AG on the bi-directional activities of 11 β HSD1 in human granulosa-lutein cells could reflect decreased paracrine inhibition of enzyme activities by progesterone.

Studies conducted on successive days of cell culture found that as the granulosa cells luteinise, the activities of 11 β HSD decrease with a concomitant increase in the

expression of 11 β HSD1 [Thurston *et al.*, 2003b]. Progesterone production also increases as human granulosa cells luteinise *in vitro* [Fowkes *et al.*, 2001]. These data are also consistent with progesterone inhibiting 11 β HSD activities in human granulosa-lutein cells.

A limitation of this study was the use of only 2 time points. 4 and 24 hour time points were used in this study to determine the acute and chronic effects of AG respectively on 11 β HSD activity and protein expression. With the benefit of hindsight and with the subsequent publication of a manuscript by Rae *et al.*, in October 2004, it would have been beneficial to extend this time course to 48 hours as maximum changes in 11 β HSD1 mRNA expression, when stimulated with IL-1 α , were detected at this time point. The studies in this Chapter have shown that the effect of AG on 11 β HSD1 activities was independent of 11 β HSD1 protein expression. Rae *et al.*, [2004] have also shown that progesterone had no effect on 11 β HSD1 mRNA synthesis. These findings suggest that the regulation of 11 β HSD activities by progesterone appears to be at a post-transcriptional and at a post-translational level. There are many possible post-translational mechanisms through which progesterone could affect 11 β HSD activities. Progesterone could be competing with cortisol/cortisone for the active site of 11 β HSD1. This would decrease the availability of active sites for cortisol/cortisone to bind to, competitively inhibiting 11 β HSD1 activities within human granulosa-lutein cells. Progesterone has been shown to competitively inhibit cortisol/cortisone metabolism in the rat myometrium [Burton and Waddell, 2002], providing one possible mechanism through which progesterone could inhibit the activities of 11 β HSD.

Another mechanism by which progesterone could decrease 11 β HSD activities could be through allosteric regulation. Progesterone could act as an allosteric effector to non-competitively inhibit 11 β HSD1 activities through binding to a hydrophobic pocket within the structure of 11 β HSD1. Structural studies have revealed that there are 2 hydrophobic regions flanking the active site of the 11 β HSD1 protein. Binding of lipids such as progesterone to these clefts could result in a conformational change of the enzyme structure, that could result in inhibition of 11 β HSD (see Chapter 7).

The third mechanism by which progesterone could affect the activities of 11 β HSD is through transmembrane signalling events. There are a number of receptors that progesterone has been shown to signal through. As well as signalling through the classical nuclear receptor, there has been evidence for progesterone acting through the cell surface gamma amino-butyric acid (GABA_A) receptor. Progesterone, its metabolites and deoxycorticosterone at physiological (low nM) concentrations have all been shown to enhance the interaction of GABA with GABA_A receptors, facilitating the open state of the GABA-gated ion channel. At higher concentrations, the steroids have been shown to directly activate the GABA_A receptor-channel complex [Lambert *et al.*, 2003]. However, studies by Peluso *et al.* [2002] have found that progesterone activation of calcium in granulosa cells is not due to the binding of GABA_A receptor channels. Moreover, they have isolated a 60kDa plasma membrane protein that binds progesterone, the binding of which is thought to mobilise intracellular calcium [Peluso *et al.*, 2002]. This could be an alternative method through which progesterone is regulating the activities of 11 β HSD. There has also

been evidence of progesterone binding to a membrane receptor that activates a number of signalling pathways including cAMP, calcium mobilisation and mitogen activated protein kinase (MAPK) [reviewed by Bramley 2003]. All of these signalling events could lead to the phosphorylation of 11 β HSD1, and change the activity of 11 β HSD.

The observed changes in 11 β HSD activities in human granulosa-lutein cells might also reflect direct effect of AG on cell survival or direct stimulation of 11 β HSD. It has been documented that AG can inhibit other cytochrome P450 enzymes of the steroidogenic pathway such as aromatase [Santen and Misbin, 1981], but has been shown to stimulate 11 β HSD activity. This could be due to AG inhibiting the synthesis of a regulator of 11 β HSD activity, rather than a direct effect of AG itself. To investigate this, studies could be carried out using a non-steroidogenic cell line (e.g. HEK293 cells) stably transfected with *HSD11B1*. The same experimental design as for human granulosa-lutein cells could be conducted using increasing concentrations of AG and investigating cortisol-cortisone conversion. This would reveal any direct stimulation inhibition of 11 β HSD1 by AG. To investigate cell survival, human granulosa-lutein cells could be stained with vital dyes such as trypan blue to assess the impact of AG on cell viability (previous attempts at this in our laboratory have excluded any significant effect of AG on cell viability). Moreover, AG has been shown to have no effect on agents which control steroidogenesis (i.e. cAMP generation and PKA activity) [D.R.E. Abayasekara, personal communication], or 3 β HSD activity in human granulosa-lutein cells Therefore the

effects of AG on 11 β HSD activity are likely to be through the inhibition of P450_{csc} and not through other non-specific mechanisms, or through effects on cell viability.

Progesterone replacement experiments could be carried out in the presence of AG to determine if the speculated effects of AG were via inhibiting progesterone synthesis. Progesterone has been shown to inhibit 11 β HSD activity in several cell types [Lopez-Bernal, 1980; Burton and Waddell, 2002] therefore it would be difficult to ascertain whether the results obtained were via endogenous progesterone synthesis being inhibited, and the addition of exogenous progesterone mimicking the speculated inhibitory effect, or through the addition of exogenous progesterone simply inhibiting 11 β HSD activity independent of its speculated effects.

RU486, a progesterone receptor antagonist [Bardon *et al.*, 1985] could be used to determine whether the effects of AG on 11 β HSD activity were via the inhibition of progesterone synthesis and hence its actions. However, a confounding factor of this study is that RU486 is also a glucocorticoid receptor antagonist [Bardon *et al.*, 1985; Rae *et al.*, 2004], therefore the results of this experiment would need to be interpreted with caution. Also, the use of a progesterone receptor antagonist presupposes that progesterone is exerting its inhibitory effects on 11 β HSD activity through intracellular mediated mechanisms, and not through possible allosteric binding to 11 β HSD1, competitive inhibition of 11 β HSD1 activity, or through the speculated membrane bound progesterone receptor. Therefore any data obtained using RU486 could be used only to complement experimental data obtained using AG and/or progesterone replacement.

A more specific mechanism of inhibiting progesterone production would be through the inhibition of 3β HSD (which converts pregnenolone to progesterone). Inhibitors such as trilostane or cyanoketone could be used to inhibit 3β HSD activity. However, pregnenolone has been shown to inhibit 11β HSD activity [A.E. Michael, personal communication]. Therefore the accumulation of pregnenolone in human granulosa-lutein cells is likely to exert inhibitory effects on 11β HSD activity. It cannot be ruled out that pregnenolone is also inhibiting 11β HSD activity in human granulosa-lutein cells, and the addition of AG could be stopping the inhibitory effects of both pregnenolone and progesterone. One way to test this would be to use a radioimmunoassay to determine the basal concentration of pregnenolone, and assess the concentration and time dependent effects of AG on pregnenolone production. That said, progesterone is the major secretory hormone of the CL, so it is likely that inhibition of 11β HSD activity is mediated predominately by progesterone.

In this study it was notable that suppression of progesterone synthesis using $100\mu\text{M}$ AG had a greater effect on the reduction of cortisone than on the oxidation of cortisol, irrespective of the duration of exposure to AG. Intuitively, if the effects of AG are solely due to removing progesterone as a competitive inhibitor of 11β HSD1 the suppression of progesterone synthesis might have been expected to have a greater effect on the low affinity 11β DH activity than on the high affinity 11KSR activity of the 11β HSD1 enzyme. However the reasons for this counterintuitive finding are not clear at the present time.

In conclusion, this study has revealed that inhibition of progesterone synthesis using AG can affect the bi-directional activities of 11 β HSD1 but not expression of 11 β HSD1 protein in human granulosa-lutein cells on day 3 of culture. The mechanisms by which progesterone affects the activities of 11 β HSD could be through competitive or non-competitive inhibition of 11 β HSD, or through a signalling event to change the phosphorylation state of the enzyme and hence its activities. Given the findings of this study, all subsequent studies investigating the endocrine regulation of 11 β HSD activities and expression were conducted in the presence of 100 μ M AG to circumvent the potential inhibition of 11 β HSD1 activities by progesterone.

**Chapter Five: Paracrine roles for eicosanoids in determining the basal activities
and protein expression of 11 β HSD1 in human granulosa-lutein cells**

**Chapter 5: Paracrine roles for eicosanoids in determining the basal activities
and protein expression of 11 β HSD1 in human granulosa-lutein cells**

5.1 Introduction

The roles of prostaglandins in the regulation of female reproduction are well documented. In the ovary, at the time of ovulation, prostaglandins are increased in the follicle, and a role for prostaglandins in luteal function has been postulated (see section 1.3). In several reproductive tissue types, prostaglandins have differential effects on the activity and expression of 11 β HSD isoforms. Hardy *et al.* [1999] have reported that in JEG-3 placental cells, PGE₂ and PGF_{2 α} can inhibit 11 β HSD2 activity. In contrast, studies by Alfaidy *et al.* [2001; 2003] have shown that PGE₂ and PGF_{2 α} can increase cortisone reduction by 11 β HSD1 within foetal membranes, instigating a positive loop between prostaglandin synthesis and cortisol regeneration from cortisone for the onset of parturition.

Although prostaglandins are known to regulate ovarian function, the role for prostaglandins in the regulation of 11 β HSD activities and expression in human granulosa-lutein cells has yet to be established. Therefore the aims of this study were:

- 1) To establish whether preferential inhibitors of PGHS2 (meclofenamic acid (MA) and NS-398) affect the activities and expression of 11 β HSD1 in human granulosa-lutein cells

- 2) To investigate the effects of exogenous prostaglandins on cortisol oxidation in human granulosa-lutein cells.

5.2 Experimental design

5.2.1 Effects of MA and NS-398 on prostaglandin production, 11 β HSD activities and 11 β HSD1 expression

To investigate the effects of MA and NS-398 on prostaglandin synthesis and on the 11 β HSD1 enzyme, human granulosa-lutein cells were isolated from follicular aspirates of women undergoing IVF-ET (section 2.2). Cells were seeded into 24 well cell culture plates at densities of 5×10^4 cells/ml media for enzyme activity studies, and 1×10^5 cells/ml for prostaglandin secretion studies. For 11 β HSD1 protein expression studies, cells were seeded into 6 well plates at a density of 1×10^6 /2ml media. Cells were cultured for 2 days to allow for luteinisation and attachment of the cells to the plates. In order to assess the effects of MA and NS-398, cells were transferred to serum-free medium.

To assess the acute and chronic effects of MA exposure on prostaglandin secretion and 11 β HSD activities, human granulosa-lutein cells were incubated with increasing concentrations of MA (0 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M, 100 μ M) for 4 and 24 hours respectively on day 3 of culture. Since MA was soluble in serum-free medium, no organic solvent was used. Where cells were treated with MA for only 4 hours, this 4 hour incubation period coincided with the final 4 hours of the 24 hours

treatment point (i.e. cells were treated either from 48-72 hours in culture, or from 68-72 hours in culture).

To assess the acute and chronic effects of NS-398 exposure on prostaglandin secretion and 11 β HSD activities, human granulosa-lutein cells were similarly incubated with increasing concentrations of NS-398 (0 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M, 100 μ M) following the same experimental protocol as for MA. The organic solvent used to solubilise NS-398 was 1% (v/v) DMSO.

To assess the ability of both MA and NS-398 to inhibit prostaglandin production, radioimmunoassays were conducted to measure the concentrations of PGE₂ and PGF_{2 α} following acute and chronic treatment with either MA or NS-398. 11 β HSD activities were assessed over 4 hours, coinciding with the last 4 hours of the 24 hour time treatment period, using a radiometric conversion assay. The steroids were chloroform extracted, resolved by TLC and cortisol-cortisone inter-conversion was quantified using a radiochromatogramme scanner (see section 2.4).

To assess changes in 11 β HSD1 protein expression, human granulosa-lutein cells were incubated for either 4 or 24 hours with the concentration of either MA or NS-398 shown to exert the maximum effects on 11 β HSD activities (100 μ M for both compounds). The effects of treatment for 4 hours coincided with the last 4 hours of the parallel 24 hour treatment incubation. Cells were extracted and proteins were separated by SDS-PAGE. 11 β HSD1 protein expression was quantified by Western blotting and densitometric analysis. Following stripping of the Western blot,

GAPDH protein expression was quantified by densitometry, and 11 β HSD1 protein expression was standardised to GAPDH expression (see sections 2.7, 2.8 and 2.9).

5.2.2 Effects of exogenous prostaglandins on cortisol oxidation

To assess whether exogenous administration of individual prostaglandins could reverse any effects of MA and NS-389 on 11 β HSD activities, human granulosa-lutein cells were incubated initially with 100 μ M MA in the presence and absence of 1 μ M PGD₂, PGE₂, PGF_{2 α} and PGI₂. A control was incubated without MA or exogenous prostaglandins for the measurement of basal 11 β HSD activity. This experimental design was subsequently repeated with the following modifications. Each prostaglandin was tested at 2 concentrations (1 μ M and 10 μ M) in the presence of either 100 μ M MA or NS-398 and in the presence of 100 μ M AG (to control for any confounding effects via changes in progesterone synthesis).

5.3 Results

5.3.1. Effect of MA on basal prostaglandin production

Treatment of human granulosa-lutein cells with MA for 4 hours inhibited both PGE₂ and PGF_{2 α} production in a concentration-dependent manner (Figure 5.1A). Maximum inhibition of prostaglandin production was observed with 100 μ M MA, inhibiting PGE₂ and PGF_{2 α} production by $64.5 \pm 17.2\%$ ($p < 0.01$) and $60.1 \pm 11.3\%$ ($p < 0.05$), respectively. Treatment with MA for 24 hours also inhibited prostaglandin synthesis in a concentration-dependent manner (Figure 5.1B). 100 μ M MA

maximally inhibited both PGE₂ and PGF_{2α} production, by approximately 50% (p>0.05 and p<0.01, respectively).

5.3.2. Effect of MA on 11βHSD activities in human granulosa-lutein cells

Treatment of human granulosa-lutein for 4 hours with MA inhibited 11βHSD activities. The effects of MA were more pronounced on cortisone reduction, with MA inhibiting 11KSR activity in a concentration-dependent manner (Figure 5.2A). Cortisol oxidation was significantly inhibited with 1μM MA by 31.9 ± 5.3% (p<0.05) and treatment with 100μM MA maximally inhibited cortisone reduction by approximately 50% of the control (p<0.01).

Treatment with MA for 24 hours also resulted in a significant inhibition of 11βHSD activities (Figure 5.2B). Following the same trend as acutely treated human granulosa-lutein cells, 100μM MA significantly inhibited cortisol oxidation over 24 hours by approximately 20% (p<0.01). Likewise, 11KSR activity was inhibited in a concentration-dependent manner, with 0.1μM MA maximally inhibiting cortisone reduction by approximately 50% (p<0.01).

Irrespective of the duration of treatment, MA appeared to more potently inhibit 11KSR activity than the oxidative activity of 11βHSD. At the maximal concentration of 100μM, MA inhibited 11KSR activity to a greater extent than 11βDH activity. Moreover, the concentration of MA required to significantly inhibit the reduction of cortisone was several orders of magnitude lower than that required to inhibit cortisol oxidation.

5.3.3. Effects of MA on 11 β HSD1 protein expression

Over both 4 and 24 hours, treatment with 100 μ M MA had no significant effect on 11 β HSD1 protein expression when standardised to GAPDH ($p > 0.05$, Figure 5.3).

5.3.4. Effects of NS-398 on basal prostaglandin production

The acute effects of NS-398 on PGE₂ and PGF_{2 α} were assessed over 4 hours. Treatment with increasing concentrations of NS-398 showed a trend for inhibiting prostaglandin synthesis (Figure 5.4A). Treatment with 100 μ M NS-398 inhibited both PGE₂ and PGF_{2 α} production by 50% ($p > 0.05$ and $p > 0.05$ respectively). Chronic treatment of human granulosa-lutein cells with NS-398 yielded similar results as acutely treated cells. PGE₂ production was maximally inhibited with 100 μ M NS-398 by $64.2 \pm 15.9\%$ ($p > 0.05$, Figure 5.4B). PGF_{2 α} production was significantly inhibited with 10 μ M NS-398 by $42.7 \pm 12.8\%$ ($p < 0.05$).

5.3.5 Effect of NS-398 on 11 β HSD activities in human granulosa-lutein cells

The acute treatment of human granulosa-lutein cells with NS-398 inhibited cortisol-cortisone inter-conversion in a concentration-dependent manner (Figure 5.5A). Cortisol oxidation was significantly inhibited with 100 μ M NS-398 by $38.3 \pm 5.8\%$ ($p < 0.05$). Maximum inhibition of cortisone reduction was achieved with 100 μ M NS-398, which inhibited cortisone reduction by $53.3 \pm 4.5\%$ ($p < 0.01$).

The chronic treatment of human granulosa-lutein cells with NS-398 revealed a similar trend as acute treatment. Again, the inhibitory effects of NS-398 were less

marked on cortisol oxidation. 100 μ M NS-398 maximally inhibited cortisol oxidation by $36.3 \pm 4.6\%$ ($p < 0.05$) and cortisone reduction by $46.9 \pm 3.7\%$ ($p < 0.001$, Figure 5.5B).

5.3.6 Effect of NS-398 on 11 β HSD1 protein expression

Both acute and chronic treatment of human granulosa-lutein cells with 100 μ M NS-398 had no effect on 11 β HSD1 protein expression ($p > 0.05$, Figure 5.6).

5.3.7 Effect of exogenous prostaglandins on cortisol oxidation

Treatment of human granulosa-lutein cells with 100 μ M MA inhibited cortisol oxidation by approximately 50% ($p < 0.001$, Figure 5.7). Co-treatment with 1 μ M PGD₂, PGE₂, PGF_{2 α} and PGI₂ did not alter the inhibitory effects of MA on cortisol oxidation.

This same experiment was repeated using each prostaglandin at a concentration of 10 μ M and in the presence and absence of 100 μ M AG. In the absence of AG, 100 μ M MA significantly inhibited cortisol oxidation ($p < 0.05$, Figure 5.8A). Even at the higher concentration of 10 μ M, the addition of exogenous prostaglandins could not overcome the inhibitory effects of 100 μ M MA on cortisol oxidation in the absence of AG ($p > 0.05$). In the presence of 100 μ M AG, the inhibitory effect of MA on cortisol oxidation was highly significant ($p < 0.001$, Figure 5.8B) and addition of exogenous prostaglandins did not overcome the inhibitory effects of MA.

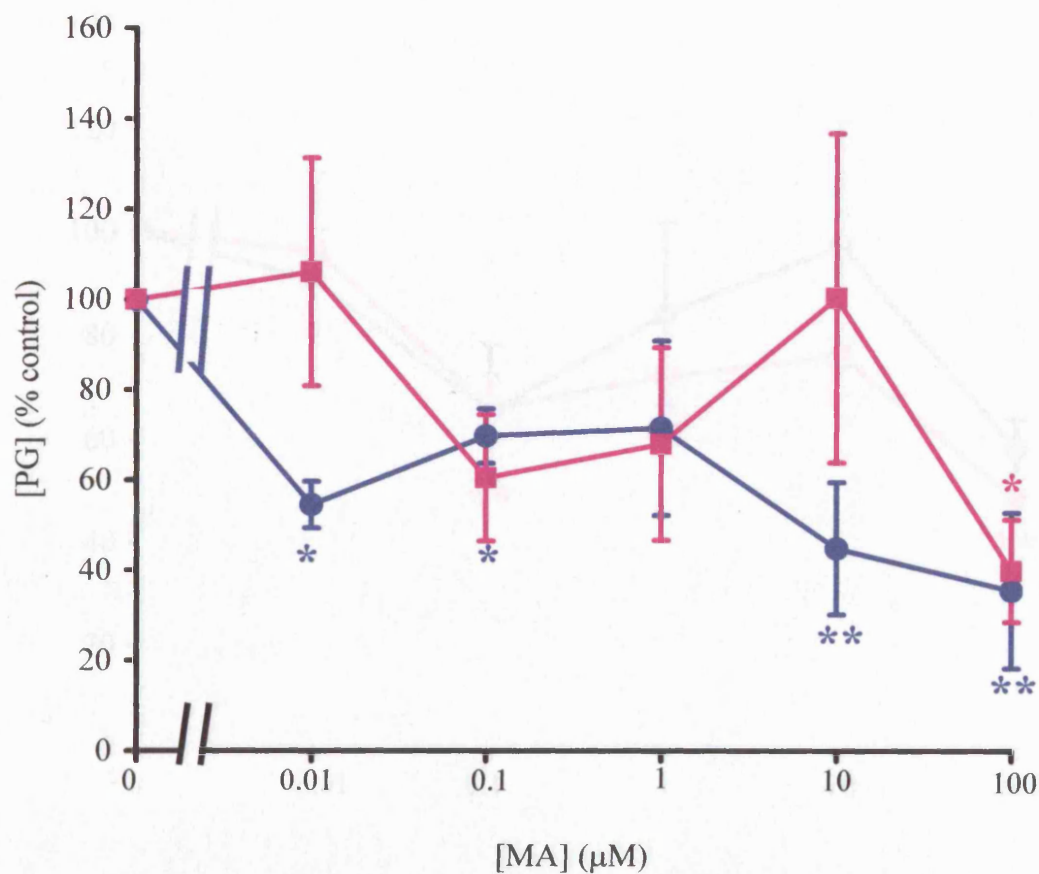


Figure 5.1A: Concentration-dependent effects of treatment with MA for 4 hours on PGE₂ and PGF_{2α} production in human granulosa-lutein cells on day 3 of culture. PGE₂= blue line, with each data point representing the mean \pm SEM for 3 independent experiment (**p<0.01 versus 0μM MA), PGF_{2α}=pink line, with each data point representing the mean \pm SEM for 4 independent experiments (*p<0.05 versus 0μM MA). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test for each prostaglandin.

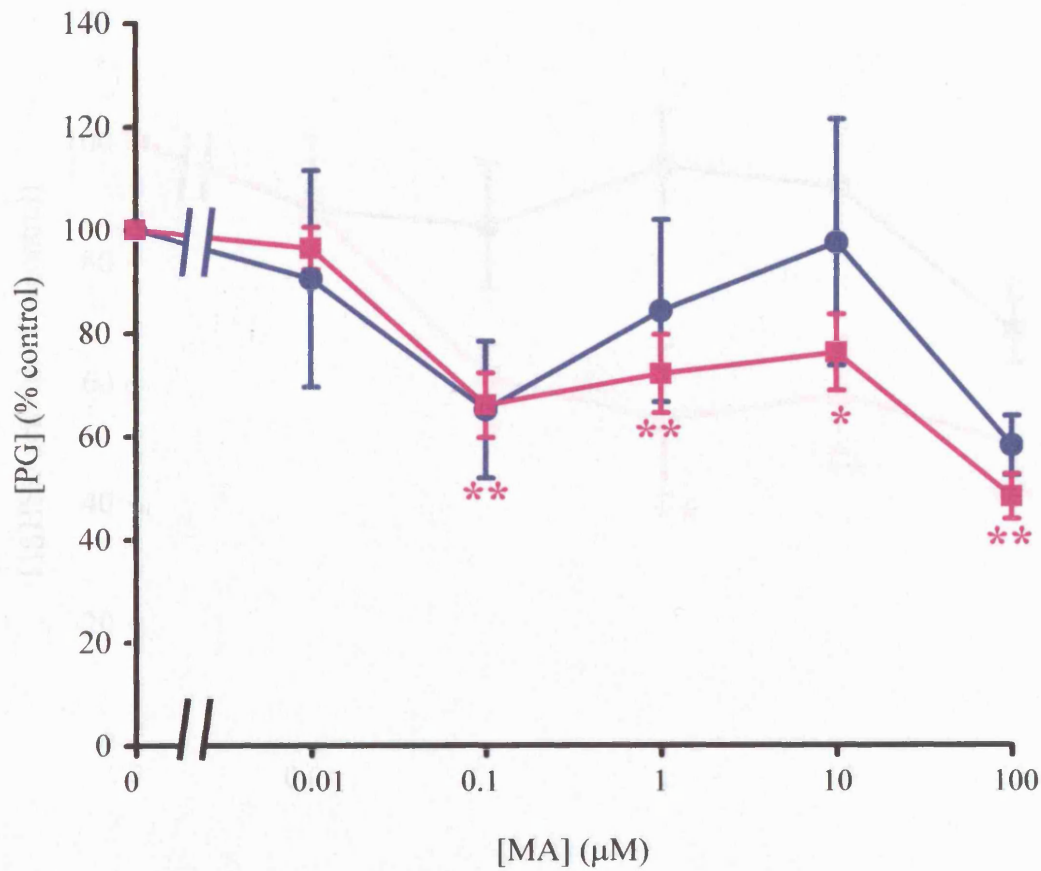


Figure 5.1B: Concentration-dependent effects of treatment with MA for 24 hours on PGE₂ and PGF_{2α} production in human granulosa-lutein cells on day 3 of culture. PGE₂=blue line, with each data point representing the mean \pm SEM for 5 independent experiments ($p>0.05$ versus 0μM MA). PGF_{2α}=pink line, with each data point representing the mean \pm SEM for 3 independent experiments (** $p<0.01$ versus 0μM MA). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test.

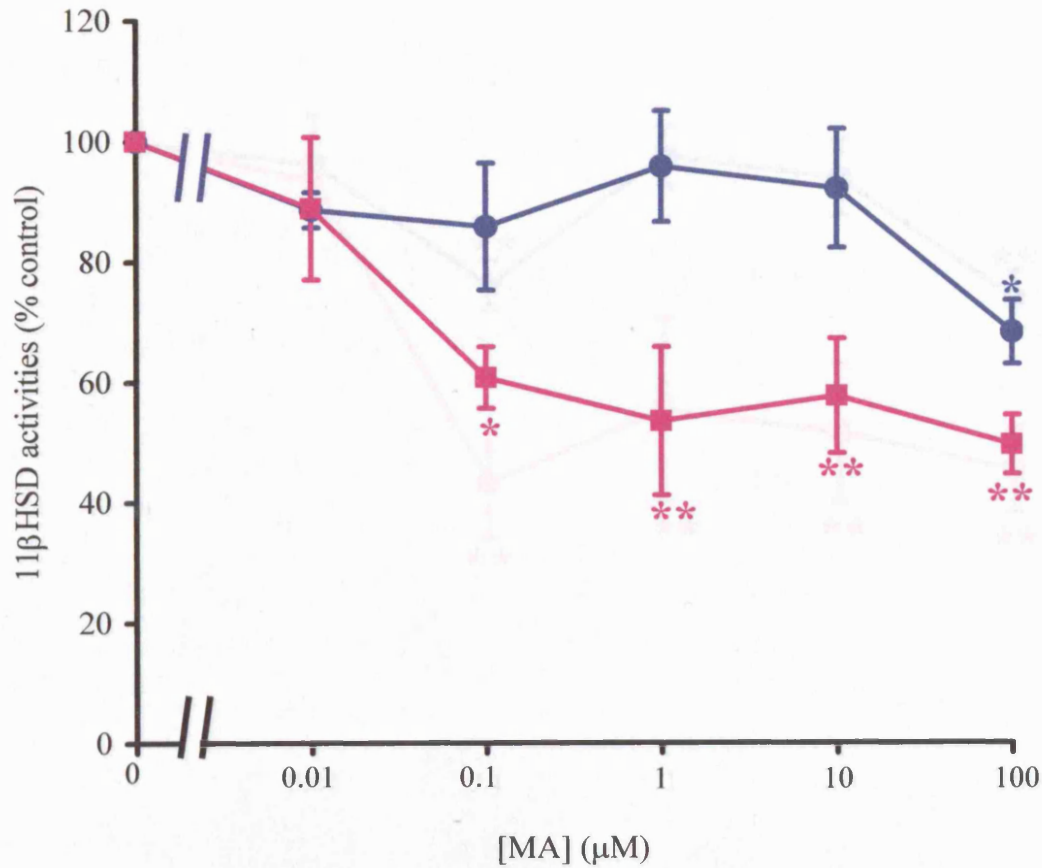


Figure 5.2A: Concentration-dependent effects of treatment with MA for 4 hours on 11βHSD activities in human granulosa-lutein cells on day 3 of culture. 11βDH activity=blue line, with each data point representing the mean \pm SEM for 5 independent experiments (* p <0.05 versus 0μM MA), and 11KSR activity=pink line, with each data point representing the mean \pm SEM for 6 independent experiments (** p <0.01 versus 0μM MA). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

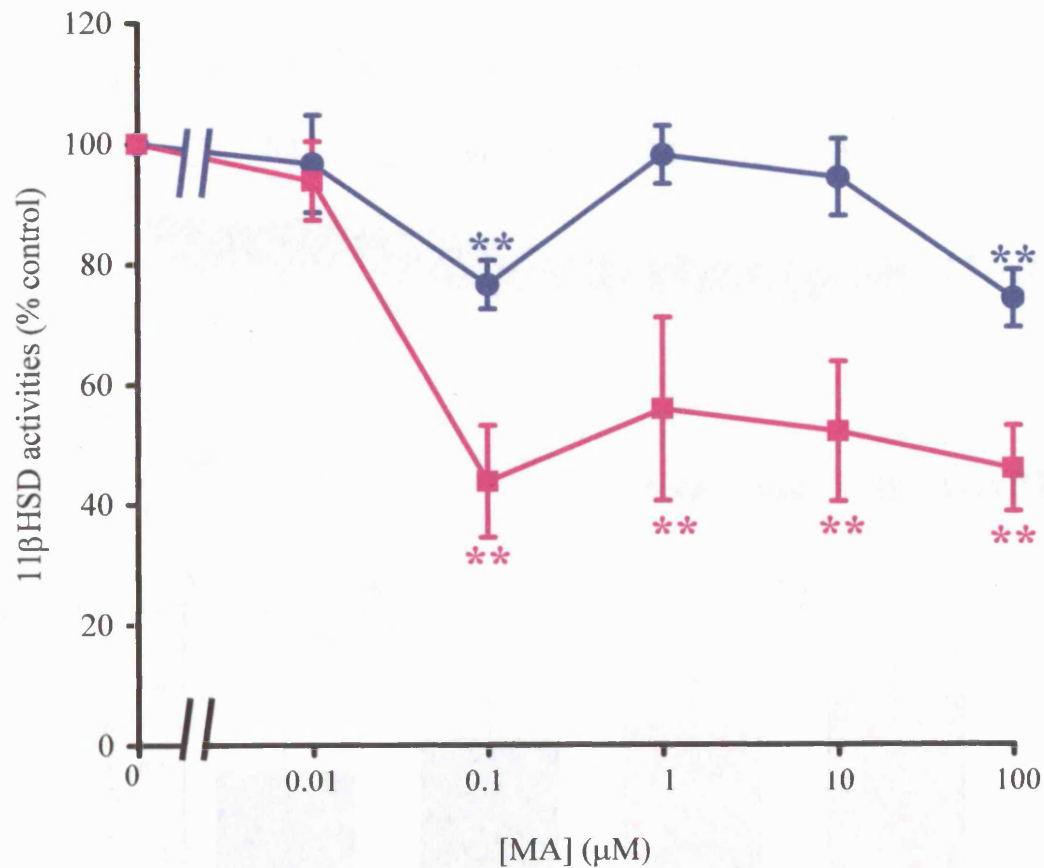


Figure 5.2B: Concentration-dependent effects of treatment with MA for 24 hours on 11βHSD activities in human granulosa-lutein cells on day 3 of culture. 11βDH activity=blue line, with each data point representing the mean \pm SEM for 5 independent experiments (** $p < 0.01$ versus 0 μM MA) and 11KSR activity=pink line, with each data point representing mean \pm SEM for 6 independent experiments (** $p < 0.01$ versus 0 μM MA). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

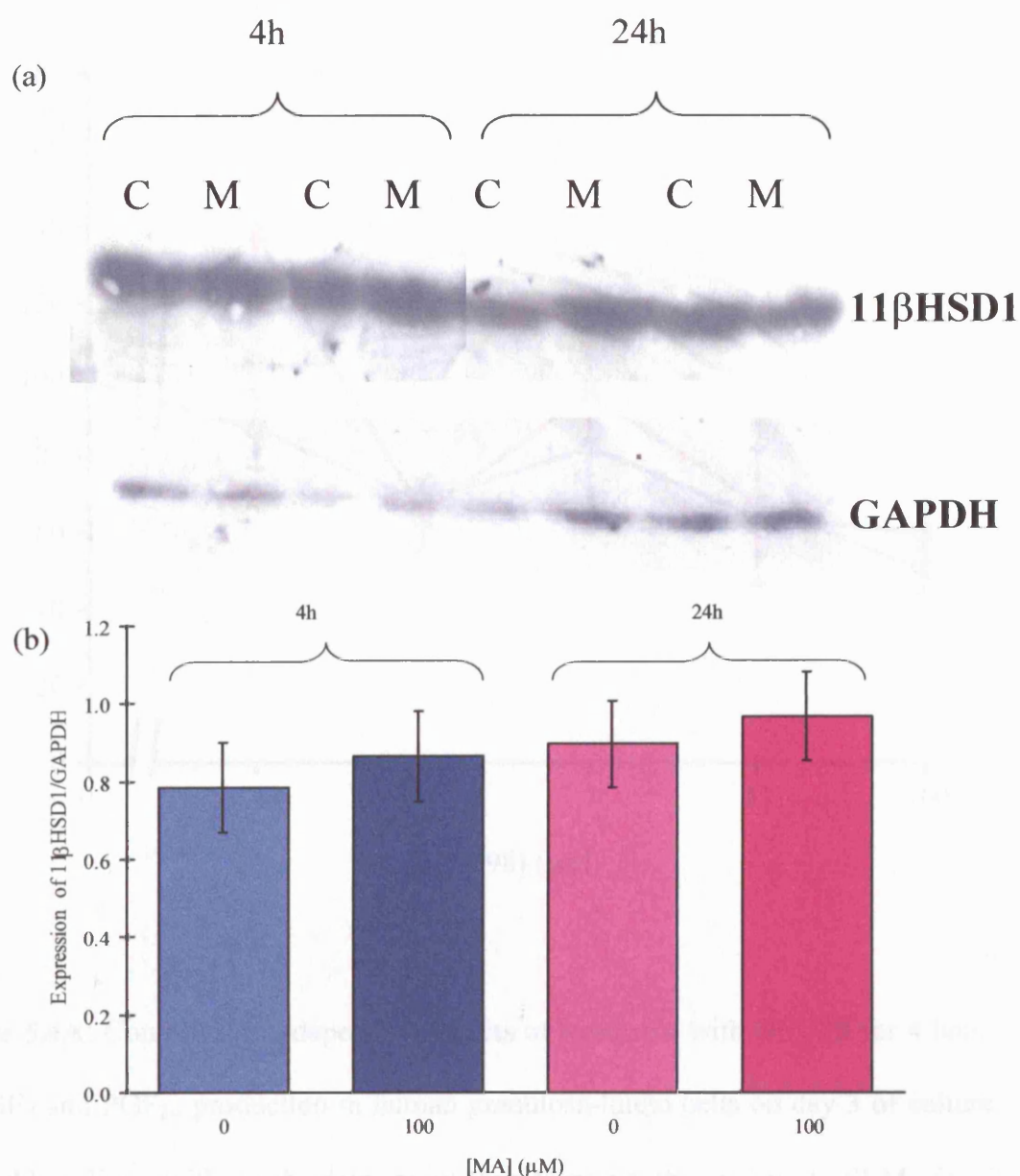


Figure 5.3: Effect of MA on expression of 11βHSD1 protein. (A) Western blot showing effect of MA on 11βHSD1 protein expression and GAPDH protein expression on day 3 of culture in human granulosa lutein cells. C represents control conditions, M represents treatment with 100μM MA. (B) Densitometric representation of effect of MA on 11βHSD1 protein expression relative to GAPDH. The blue bars represent experiments carried out over 4 hours. The pink bars represents experiments carried out over 24 hours. Hatched bars represent control, and solid bars represent hGL cells treated with 100μM MA. Each data point represents the mean ± SEM for 3 independent experiments, ($p > 0.05$ versus 0μM MA). Means compared at each time point using unpaired T-test.

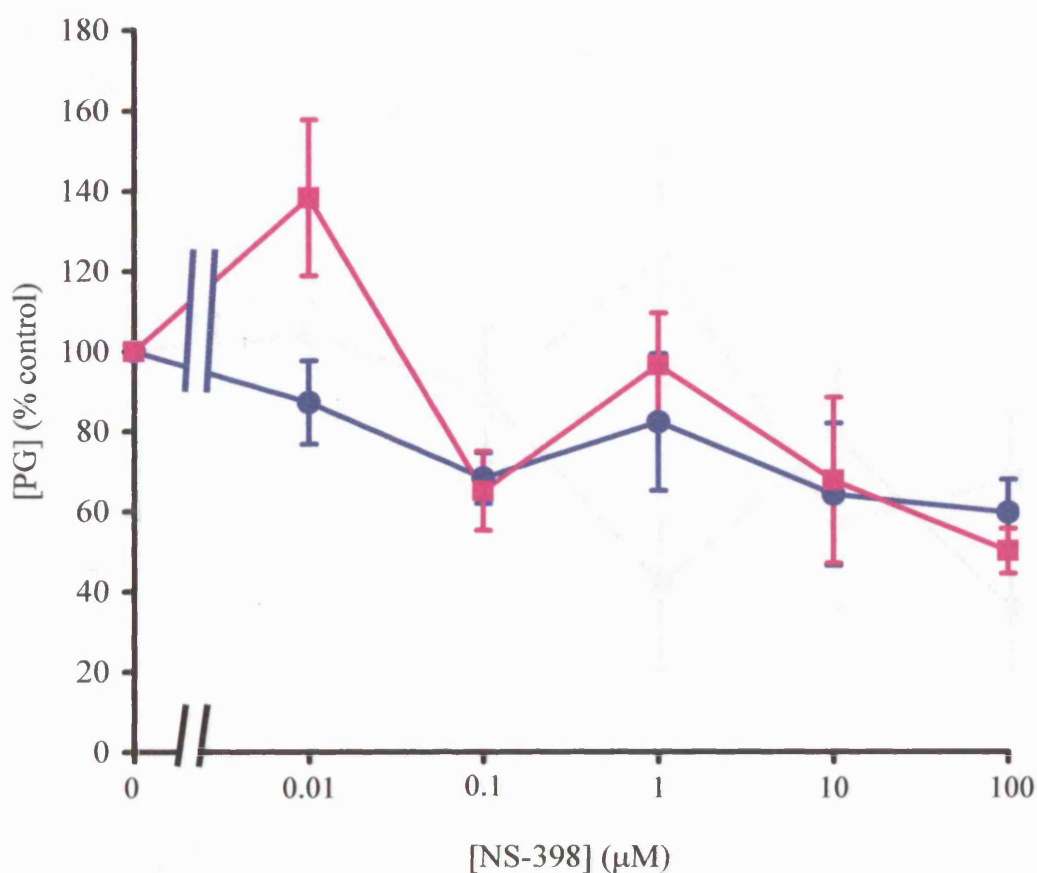


Figure 5.4A: Concentration-dependent effects of treatment with NS-398 for 4 hours on PGE₂ and PGF_{2α} production in human granulosa-lutein cells on day 3 of culture. PGE₂=blue line, with each data point representing the mean ± SEM for 3 independent experiment ($p > 0.05$ versus 0 μM NS-398), and PGF_{2α}=pink line, with each data point representing the mean ± SEM for 6 independent experiments ($p > 0.05$ versus 0 μM NS-398). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

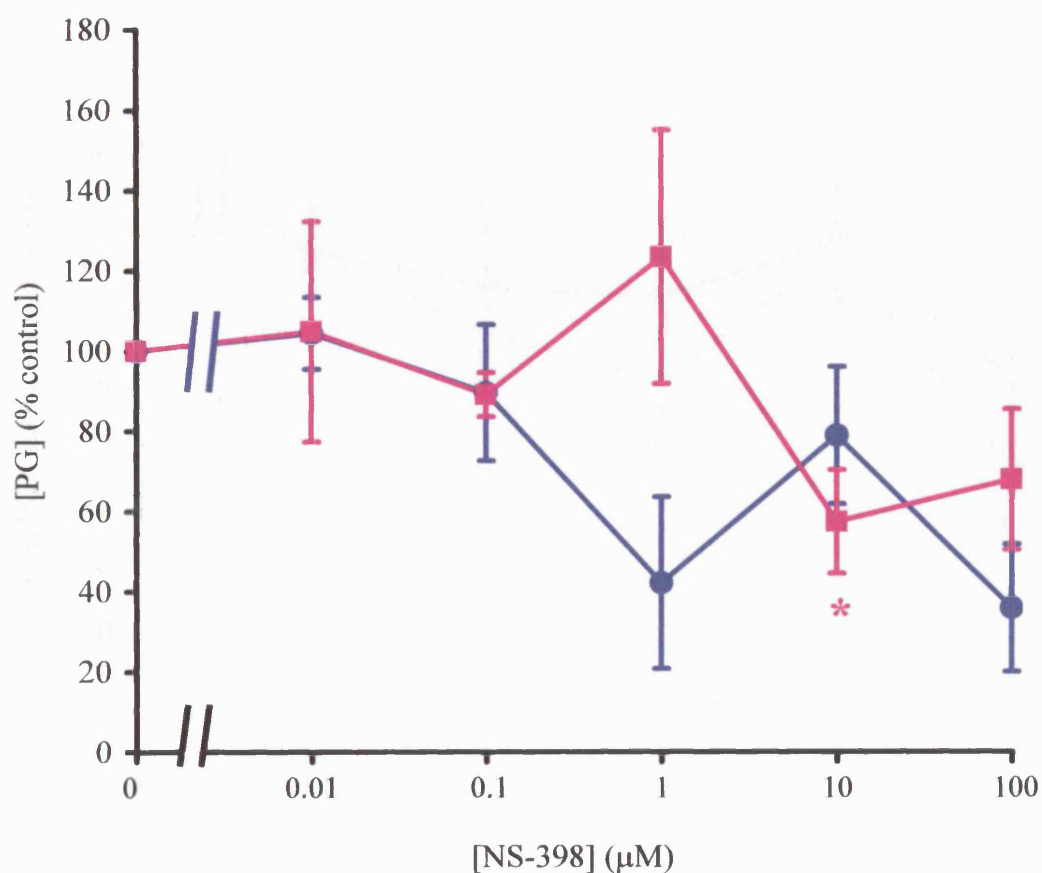


Figure 5.4B: Concentration-dependent effects of treatment with NS-398 for 24 hours on PGE₂ and PGF_{2α} production in human granulosa-lutein cells on day 3 of culture. PGE₂=blue line, with each data point representing the mean \pm SEM for 5 independent experiments ($p>0.05$ versus 0μM NS-398) and PGF_{2α}=pink line, with each data point representing the mean \pm SEM for 5 independent experiments (* $p<0.05$ versus 0μM NS-398). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

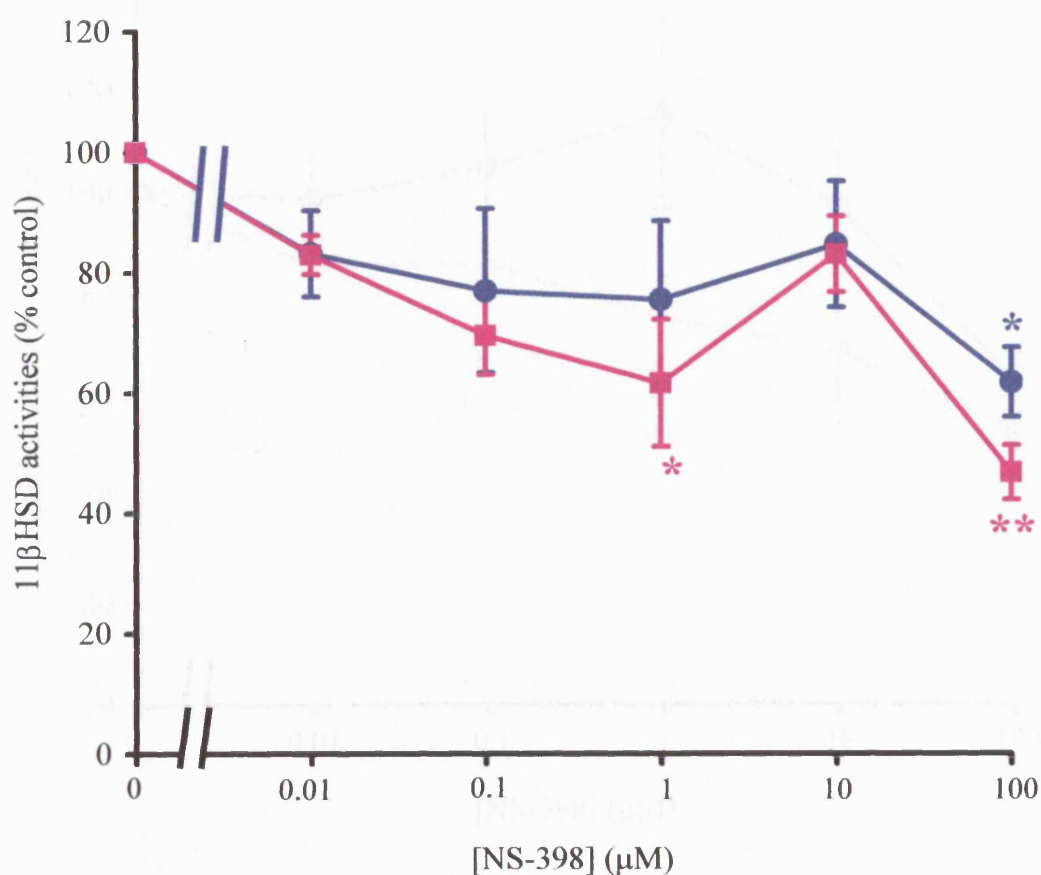


Figure 5.5A: Concentration-dependent effects of treatment with NS-398 for 4 hours on 11βHSD activities in human granulosa-lutein cells on day 3 of culture. 11βDH activity=blue line, with each data point representing the mean \pm SEM for 4 independent experiments (* p <0.05 versus 0μM NS-398) and 11KSR activity=pink line, with each data point representing the mean \pm SEM for 3 independent experiments (** p <0.01 versus 0μM NS-398). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

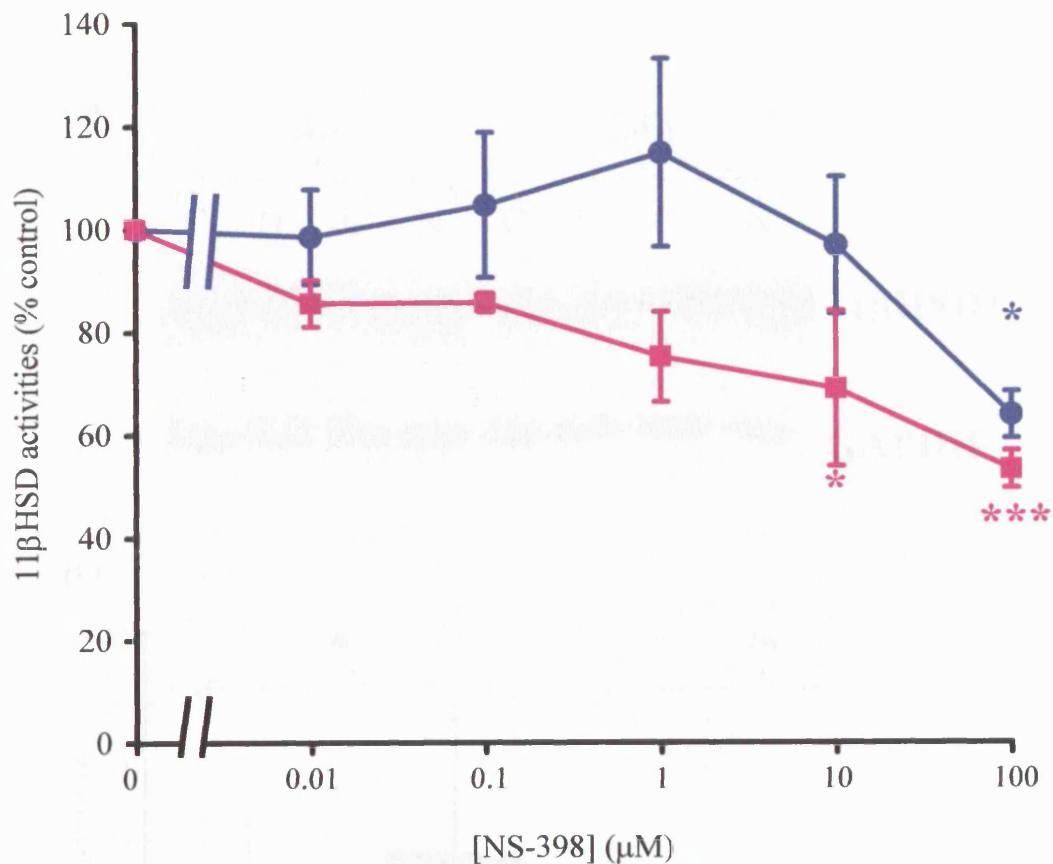


Figure 5.5B: Concentration-dependent effects of NS-398 on 11βHSD activities in human granulosa-lutein cells on day 3 of culture over 24 hours. 11βDH activity=blue line, with each data point representing mean \pm SEM for 4 independent experiments (* p <0.05 versus 0μM NS-398), and 11KSR activity=pink line, with each data point representing the mean \pm SEM for 3 independent experiments (*** p <0.001 versus 0μM NS-398). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

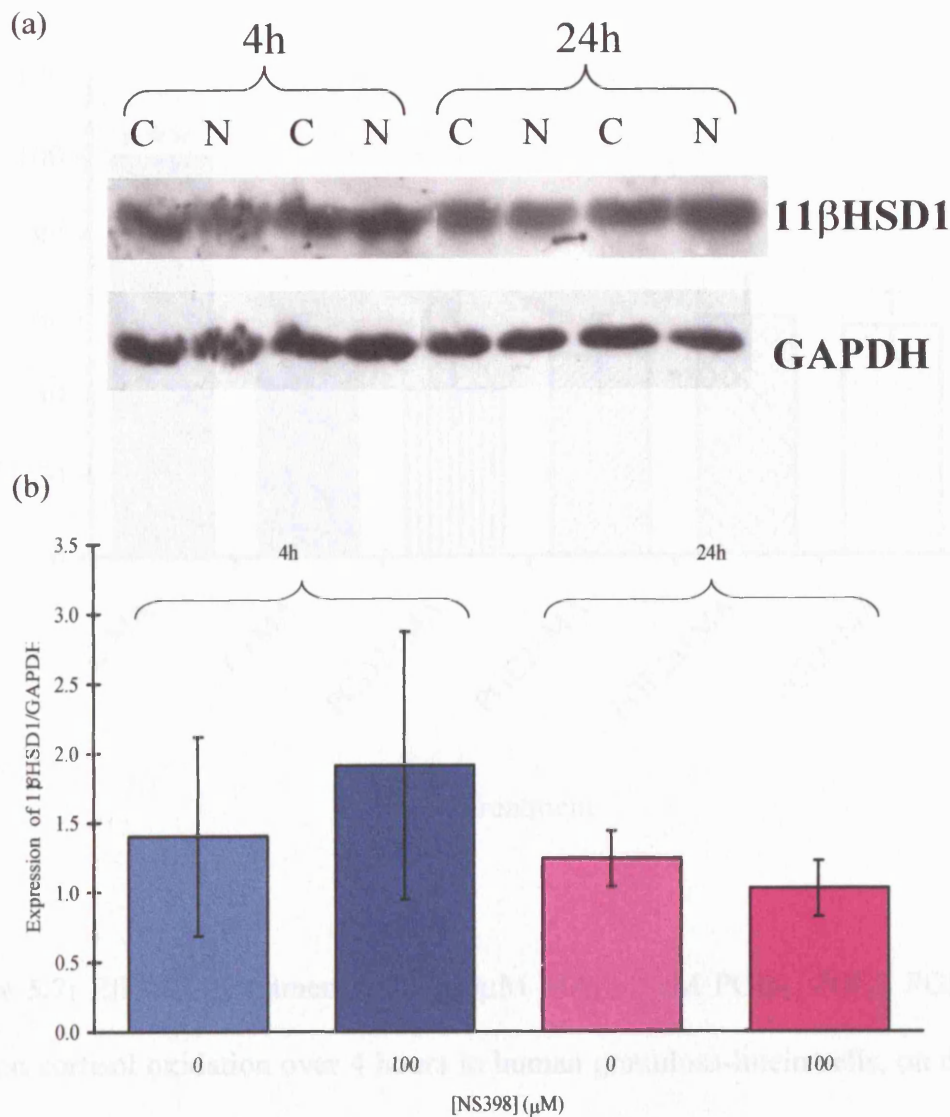


Figure 5.6: Effect of NS-398 on expression of 11βHSD1 protein expression. (A) Western blot showing effect of NS-398 on 11βHSD1 protein expression relative to GAPDH on day 3 of culture in human granulosa lutein cells. C represents control conditions, N represents treatment with 100μM NS-398. (B) Densitometric representation of effect of NS-398 on 11βHSD1 protein expression relative to GAPDH. The blue bars represent experiments carried out over 4 hours. The pink bars represents experiments carried out over 24 hours. Hatched bars represent control, and solid bars represent hGL cells treated with 100μM NS-398. Each data point represents the mean \pm SEM of 3 independent experiments ($p > 0.05$ versus 0μM NS-398). Means compared at each time point using unpaired T-test.

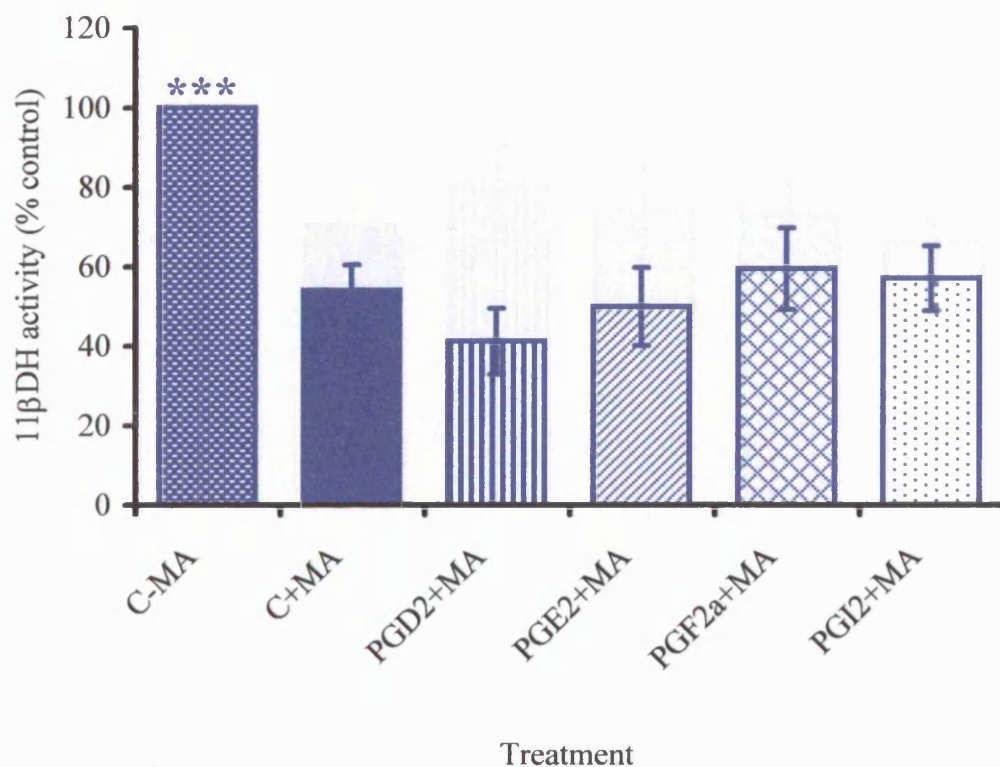


Figure 5.7: Effect of treatment with 100μM MA ± 1μM PGD₂, PGE₂, PGF_{2α} and PGI₂ on cortisol oxidation over 4 hours in human granulosa-lutein cells, on day 3 of culture. Solid panel represents control with 100μM MA. Hatched lines represent the absence of 100μM MA. Each data point represents the mean ± SEM for 4 independent experiments (**p<0.01 versus C+MA). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

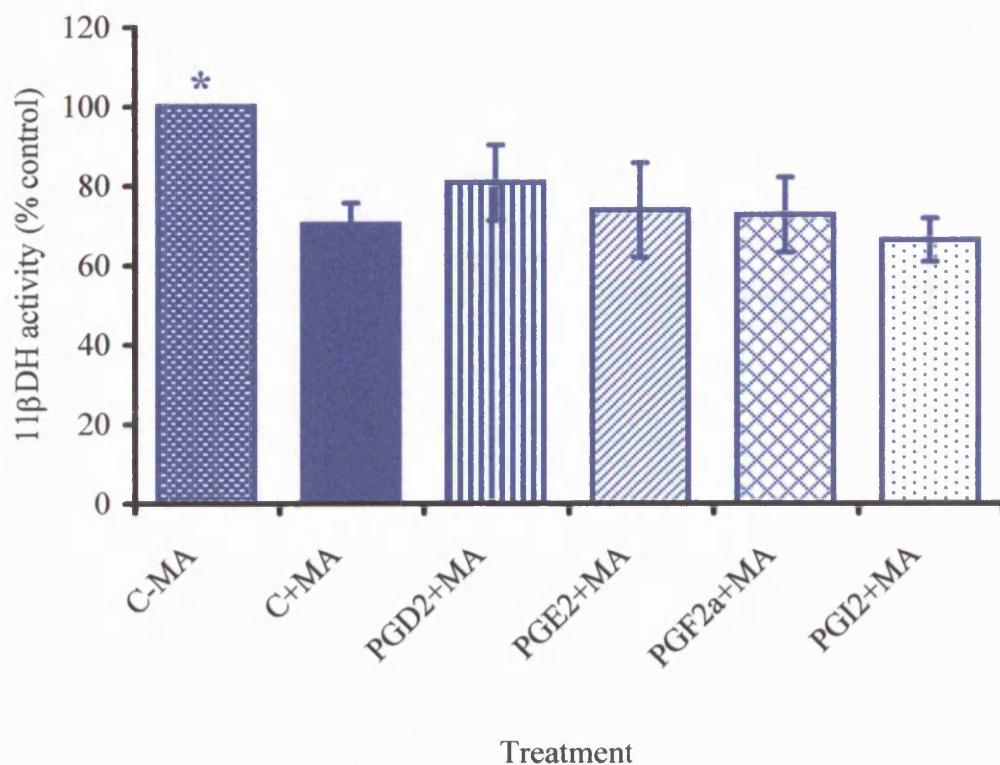


Figure 5.8A: Effect of treatment with 100μM MA ± 10μM PGD₂, PGE₂, PGF_{2α} and PGI₂ on cortisol oxidation over 4 hours in human granulosa-lutein cells, on day 3 of culture in the absence of AG. Solid panel represents control in the presence of 100μM MA. Hatched lines represent the absence of 100μM MA. Each data point represents the mean ± SEM for 4 independent experiments (*p<0.05 versus C+MA). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

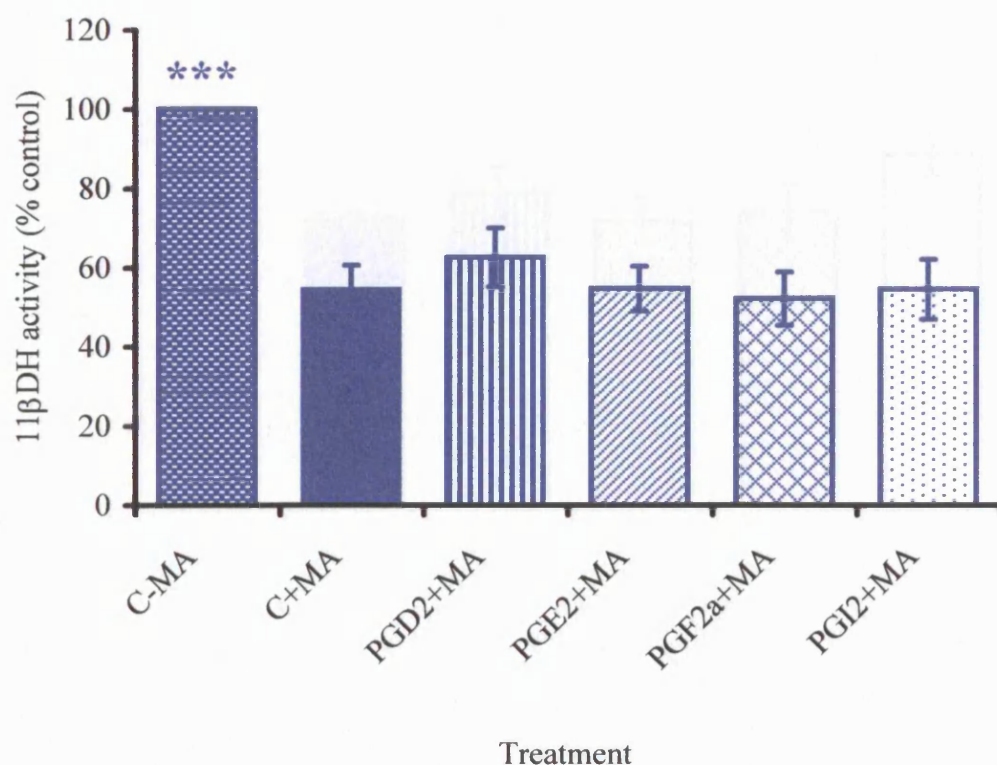


Figure 5.8B: Effect treatment with 100μM MA ± 10μM PGD₂, PGE₂, PGF_{2α} and PGI₂ on cortisol oxidation over 4 hours in human granulosa-lutein cells, on day 3 of culture in the presence of AG. Solid panel represents control with 100μM MA. Hatched lines represent the absence of 100μM MA. Each data point represents the mean ± SEM for 4 independent experiments (***p<0.001 versus C+MA). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

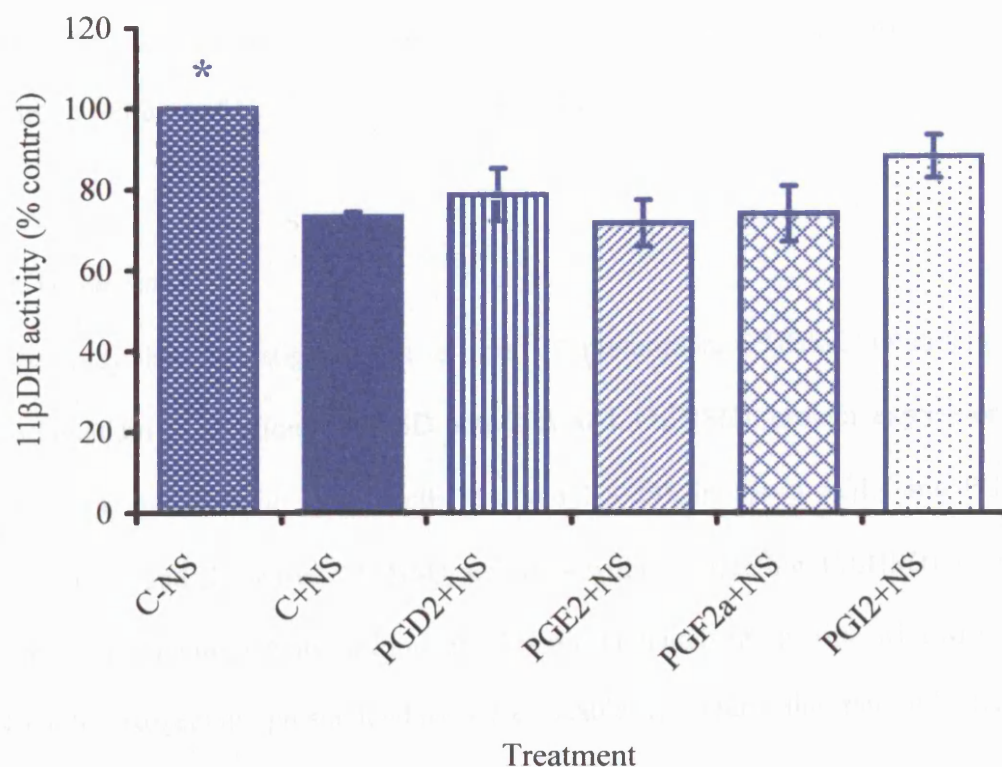


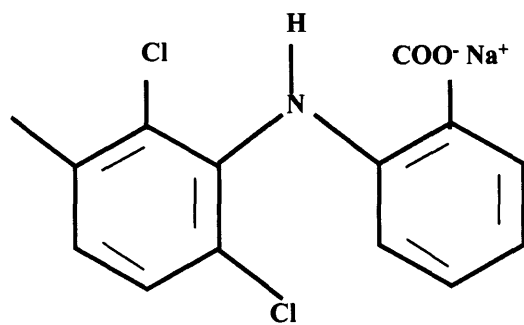
Figure 5.9: Effect of treatment with 100μM NS-398 ± 10μM PGD₂, PGE₂, PGF_{2α} and PGI₂ on cortisol oxidation over 4 hours in human granulosa-lutein cells, on day 3 of culture. Experiments were conducted in the presence of AG. Solid panel represents control without 100μM NS-398. Hatched lines represent the presence of 100μM NS-398. Each data point represents the mean ± SEM for 4 independent experiments (*p<0.05 versus C+NS-398). Data analysed with repeated measures one-way ANOVA followed by Dunnett's multiple comparison test

This same experimental design was repeated using 100 μ M NS-398 in place of MA. In the presence of AG, NS-398 inhibited cortisol oxidation by $27.8 \pm 1.2\%$ ($p < 0.05$, Figure 5.9). Co-treatment with exogenous prostaglandins could not overcome the inhibitory effects of NS-398 on cortisol oxidation.

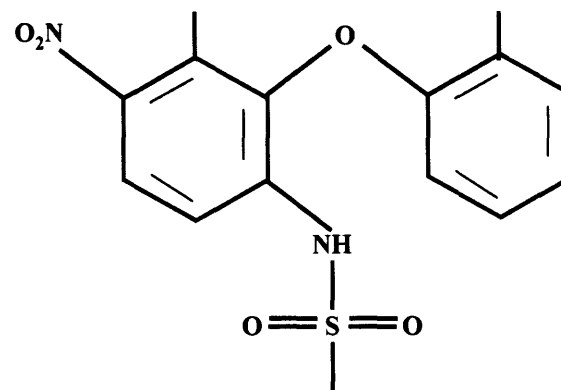
5.4. Discussion

This study has investigated the effects of preferential PGHS-2 inhibitors on prostaglandin production, 11 β HSD activities and 11 β HSD1 protein expression in human granulosa-lutein cells. Both MA and NS-398 inhibited PGE₂ and PGF_{2 α} production, and decreased 11 β HSD activities without affecting 11 β HSD1 protein expression. Having suppressed endogenous prostaglandin production with MA and NS-398, exogenous prostaglandins were unable to return the rate of cortisol oxidation to control levels.

Differences were observed in the potencies of MA and NS-398 for inhibiting prostaglandin production. There are several explanations for these differences. Firstly the solubility of MA and NS-398 is different due to structural differences between the 2 compounds (Figure 5.10). MA is more hydrophilic and is soluble in aqueous media, whereas NS-398 is less hydrophilic and insoluble in aqueous solution such that it requires an organic solvent to increase its solubility in medium. These structural differences could affect the rate of uptake of the compounds into the cell. Moreover, once entering the cell these structural differences could result in a difference in the solubility of the compounds within the cytosol, which would also



Meclofenamic Acid



NS-398

Figure 5.10: Structures of Meclofenamic acid and NS-398. Adapted from Cayman Chemicals, USA

affect the rate at which the compounds are transported out of the cell. These physical factors could result in differential effects of MA and NS-398 on prostaglandin synthesis and possibly on 11 β HSD activities.

Another difference could be the specificity of MA and NS-398 for PGHS2. MA has a K_i of 1.5 μ M for PGHS2, and a K_i of 9.7 μ M for PGHS1 [Laneuville *et al.*, 1994]. Hence at concentrations of 10 μ M or 100 μ M, the effects of MA could reflect inhibition of PGHS1 as well as inhibiting PGHS2. NS-398 is a more selective inhibitor of PGHS2, with a K_i for PGHS2 of 1.77 μ M and a K_i for PGHS1 of 75 μ M [Kargman *et al.*, 1996]. These differences in inhibitory constants could explain why MA is more effective at suppressing prostaglandin production since it inhibits both PGHS2- and PGHS1-mediated prostaglandin synthesis with near equal affinity.

MA and NS-398 also display different potencies in inhibiting the activities of 11 β HSD. The simplest explanation of this finding is that MA and NS-398 could have direct inhibitory effects on the activities of 11 β HSD. However, previous studies by a former graduate student in the laboratory had established that 11 β HSD activities in human granulosa-lutein cells could also be inhibited by indomethacin: a PGHS1 and PGHS2 inhibitor which has a notably different chemical structure to MA and NS-398. The capacity of structurally dissimilar inhibitors of the PGHS enzymes to inhibit 11 β HSD activities in human granulosa-lutein cells makes it unlikely that these compounds act by direct effects on the 11 β HSD enzymes.

Were the effects of MA and/or NS-398 on cortisol-cortisone inter-conversion to reflect direct actions, one would expect equivalent effects on bi-directional enzyme activities. If anything, a given drug should be more effective at non-specifically inhibiting the low affinity oxidative activity of 11 β HSD1 versus the high affinity reductase activity of this enzyme. However, MA was more potent in inhibiting the 11KSR activity of human granulosa-lutein cells than the 11 β DH activity, both in terms of the degree of maximum inhibition of enzyme activity achieved at 100 μ M MA, and the concentration of MA required to achieve significant inhibition (0.1-1 μ M MA for 11 KSR activity versus 100 μ M MA for 11 β DH activity). Hence it seems highly unlikely that the decrease in the 11 β DH and 11KSR activities in human granulosa-lutein cells reflect non-specific, direct inhibition of 11 β HSD1 by MA or NS-398.

The suppression of local prostaglandin synthesis with MA and NS-398 had differential effects on cortisol oxidation and cortisone reduction. Cortisone reduction was more sensitive to inhibition at lower concentrations of MA and NS-398 than cortisol oxidation. This could reflect differences in the substrate rates of cortisol oxidation versus cortisone reduction. Control rates of cortisol oxidation were more than double the control rate of cortisone reduction (8-10 pmol cortisol oxidised/5 x 10⁴ cells.4h versus 3-4 pmol cortisone reduced/5 x 10⁴ cells.4h). Due to this difference in absolute substrate conversion, it may be easier to detect a change in 11KSR activities than a change in 11 β DH activity. E.g., a decrease in the level of substrate metabolism by the same unit of 1pmole/5 x 10⁴ cells.4h would represent a

25% decrease for 11KSR activity, but only around a 10% decrease in 11 β DH activity.

The more likely explanation of these findings is that the effect of MA and NS398 (and of indomethacin) on 11 β HSD activities reflected changes in the pattern of AA metabolism to eicosanoids following inhibition of PGHS1/2. Since the concentrations of MA and NS-398 required to inhibit 11 β HSD activities coincides with the concentrations of MA and NS-398 required to significantly inhibit prostaglandin synthesis, the decrease in cortisol-cortisone inter-conversion by human granulosa-lutein cells could reflect a positive autocrine/paracrine role for prostaglandins in stimulating 11 β HSD1 activities in human granulosa-lutein cells.

The addition of exogenous prostaglandins to human granulosa-lutein cells in the presence of either MA or NS-398 failed to restore the rate of cortisol oxidation to control levels even when prostaglandins were added at concentrations of 10 μ M. There are several explanations for this finding. Firstly, addition of exogenous extracellular prostaglandins may not replicate the effects of prostaglandins endogenously produced within cells. In ovarian cells, multiple prostanoids are synthesised, and multiple receptor subtypes are expressed [Narumiya *et al.*, 1999]. Hence the addition of individual specific prostaglandins may not be able to fully overcome the effects of inhibiting the synthesis of all prostaglandins.

Secondly, the failure of exogenous prostaglandins to restore rates of cortisol oxidation could reflect the abilities of MA and NS-398 to inhibit other pathways of

AA metabolism. MA is known to inhibit the 5- and -15 lipoxygenase pathway [Stadler *et al.*, 1994] and other non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, have also been shown to inhibit other pathways of AA metabolism in the ovary [Downey *et al.*, 1998]. The inhibition of 5- and 15-lipoxygenase by MA could account for MA more potently inhibiting 11 β HSD activities than NS-398, if lipoxygenase products (e.g. leukotriene B₄) elevate the basal levels of cortisol-cortisone inter-conversion in human granulosa-lutein cells. To confirm the specificity of MA and NS-398 for inhibiting just prostaglandin production, enzyme linked immunoassays (ELISAs) could be conducted to monitor products of the lipoxygenase pathway such as leukotrienes and HETEs in the presence of MA and NS-398.

Finally the effects of MA and NS-398 on 11 β HSD activities in human granulosa-lutein cells could involve the re-direction of the flux of AA metabolism through other associated pathways. The inhibition of the PGHS enzymes by MA and/or NS-398 will increase the flux of AA through the lipoxygenase and epoxygenase pathways. Hence, the decrease of cortisol oxidation or cortisone reduction achieved by MA and NS-398 could implicate lipoxygenase and /or epoxygenase products as inhibitors of 11 β HSD1 in human granulosa-lutein cells.

In conclusion, the findings reported in this Chapter has implied a role for eicosanoids in regulating the basal activities of 11 β HSD in human granulosa-lutein cells without affecting 11 β HSD1 protein expression. Hence, the studies reported in Chapter 6 will

explore the potential mediatory roles for prostaglandins in the stimulation of ovarian
11 β HSD activities by hCG and IL-1 β .

Chapter Six: Role of prostaglandins in mediating the effects of hCG and IL-1 β
on the activity and expression of 11 β HSD1 in human granulosa-lutein cells

Chapter 6: Role of prostaglandins in mediating the effects of hCG and IL-1 β on the activity and expression of 11 β HSD1 in human granulosa-lutein cells

6.1 Introduction

As previously described in section 1.3 and 5.1, prostaglandins play a key role in ovarian function and have been shown to regulate the activity and expression of 11 β HSD in various tissue types. Inflammatory cytokines have also been implicated in the regulation of 11 β HSD1 activity and expression in several tissue types. Both TNF- α and IL-1 β can increase 11 β HSD1 activity and mRNA expression in human amnion [Sun and Myatt 2003], pre-adipocytes [Friedberg *et al.*, 2003], osteoblasts [Cooper *et al.*, 2001], glomerular mesangial cells [Escher *et al.*, 1997], and in the ovary [Tetsuka *et al.*, 1999 Yong *et al.*, 2002; Rae *et al.*, 2004]. Gonadotrophins have also been shown to regulate the activity and expression of 11 β HSD isoforms in rat Leydig cells [Gao *et al.*, 1997] and granulosa cells [Tetsuka *et al.*, 1999b].

It has been long established that ovulation is an inflammatory cascade that is orchestrated and modulated by inflammatory prostaglandins and cytokines, initiated by the gonadotrophin surge [Espey 1980]. Therefore the aims of this study were:

- 1) To determine the effects hCG and IL-1 β on prostaglandin production, 11 β HSD1 activities and 11 β HSD1 protein expression in human granulosa-lutein cells
- 2) To establish the effects of co-treatment with MA and NS-398 on hCG and IL-1 β control of 11 β HSD activities and expression

6.2 Experimental design

6.2.1 Acute and chronic effects of hCG and IL-1 β on prostaglandin production and 11 β HSD activities

Human granulosa-lutein cells, isolated from follicular aspirates of women undergoing IVF-ET, were seeded into 24 well cell culture plates at a density of 1×10^5 cells/ml for prostaglandin production studies, and 5×10^4 cells/ml for 11 β HSD activity studies, into serum-supplemented medium (see section 2.2). Cells were cultured for 2 days to allow for luteinisation and for attachment to the cell culture plates. Cells were then transferred to serum-free medium to assess the effects of hCG and IL-1 β on prostaglandin production, and the bi-directional activities of 11 β HSD1.

To assess the acute and chronic effects of hCG and IL-1 β exposure, human granulosa-lutein cells were incubated with medium alone (control), 100ng/ml hCG or 10ng/ml IL-1 β for 4 and 24 hours. The acute effects of hCG and IL-1 β were determined over 4 hours, coinciding with the last 4 hours of the 24 hour treatment incubations. These studies were conducted in the presence and absence of 100 μ M AG (to inhibit progesterone responses to hCG and/or IL-1 β).

To determine the effects of hCG and IL-1 β on prostaglandin production, PGE₂ and PGF_{2 α} concentrations were assessed by RIA (see section 2.3). In parallel cell culture plates, 11 β HSD activities were assessed by radiometric conversion assayed during the final 4 hours of each experiment. Cortisol-cortisone inter-conversion was determined by radiometric conversion assay. To terminate the reaction, spent medium was transferred to borosilicate glass tubes, the steroids were chloroform

extracted, and resolved by TLC. Cortisol-cortisone conversion was quantified using a radiochromatogramme scanner (see section 2.4).

6.2.2 Concentration-dependent effects of IL-1 β and hCG on prostaglandin synthesis and 11 β HSD1 activities

To investigate the concentration-dependent effects of hCG, cells were incubated with increasing concentrations of hCG (0, 1, 3, 10, 30, 100ng/ml) in the presence and absence of 100 μ M MA or 100 μ M NS-398 for 24 hours. To minimise the confounding effects of progesterone, these experiments were conducted in the presence of 100 μ M AG. The concentration-dependent effects of IL-1 β were investigated using increasing concentrations of IL-1 β (0, 3, 1, 3, 10ng/ml) in the presence and absence of 100 μ M MA or 100 μ M NS-398, and in the presence of 100 μ M AG for 24 hours.

The concentration dependent effects of hCG and IL-1 β on prostaglandin production were assessed by RIA of PGE₂ and PGF_{2 α} . Cortisol-cortisone inter-conversion was assessed over the last 4 hours of the 24 hour treatment period using a radiometric conversion assay. Steroids were chloroform extracted, resolved by TLC and quantified using a radiochromatogramme scanner.

6.2.3 Effects of hCG and IL-1 β on 11 β HSD1 protein expression

For the assessment of changes in 11 β HSD1 protein expression, human granulosa-lutein cells were seeded into 6 well plates at a density of 1 x 10⁶ cells/ml in serum-

supplemented medium. Cells were transferred into serum-free medium and incubated for 24 hours with those concentrations of hCG (100ng/ml) and IL-1 β (10ng/ml) shown to exert the greatest effects on 11 β HSD activities. Cells were treated in the presence or absence of 100 μ M MA and in the presence of 100 μ M AG. Cells were extracted and proteins separated by SDS-PAGE. 11 β HSD1 protein expression was quantified through Western blot and densitometric analysis. Following stripping of the Western blot, GAPDH protein expression was quantified by densitometry. 11 β HSD1 protein expression was standardised to GAPDH expression (see sections 2.7, 2.8 and 2.9).

6.3 Results

6.3.1 Effects of hCG and IL-1 β on PGE₂ and PGF_{2 α} production

In absence of AG, treatment of human granulosa-lutein cells with either hCG (100ng/ml) or IL-1 β (10ng/ml) for 4 hours had no significant effect on PGE₂ (Figure 6.1A) and PGF_{2 α} (Figure 6.1B) production ($p>0.05$) irrespective of the presence or absence of MA. Likewise, in the presence of 100 μ M AG, acute treatment with either hCG or IL-1 β again had no significant effect on PGE₂ (Figure 6.2A) and PGF_{2 α} (Figure 6.2B) production, irrespective of the presence or absence of MA ($p>0.05$).

In the absence of AG, treatment of human granulosa-lutein cells for 24 hours with 10ng/ml IL-1 β tended to increase PGE₂ production, ($p>0.05$, Figure 6.3A) and significantly increased PGF_{2 α} production to $166.7 \pm 6.5\%$ of control ($p<0.05$, Figure 6.3B). Co-treatment with MA abolished both of the prostaglandin responses.

Treatment of human granulosa-lutein for 24 hours cells with 100ng/ml hCG had no effect on either PGE₂ or PGF_{2α} production, irrespective of the presence or absence of MA.

In the presence of AG, treatment of human granulosa-lutein cells for 24 hour with hCG tended to increase PGE₂ production, and IL-1β significantly increased PGE₂ production to $144.2 \pm 3.4\%$ of control ($p < 0.05$, Figure 6.4A). IL-1β also tended to increase PGF_{2α} production by up to $186.5 \pm 46.7\%$ of control ($p > 0.05$, Figure 6.4B). Co-treatment with MA was shown to inhibit these responses. Treatment with 100ng/ml hCG for 24 hours had no effect on PGF_{2α} production by human granulosa-lutein cells ($p > 0.05$, Figure 6.4B).

6.3.2 Acute effects of hCG and IL-1β on 11βHSD activities

In the absence of AG and in the absence of MA, treatment of human granulosa-lutein cells for 4 hours with either 100ng/ml hCG or 10ng/ml IL-1β stimulated cortisol oxidation to $146.6 \pm 23.5\%$ ($p < 0.05$) and $147.3 \pm 16.0\%$ ($p < 0.01$, Figure 6.5A) of control enzyme activity respectively. Co-treatment with 100μM MA abolished the stimulation of 11β-dehydrogenase activity by both hCG and IL-1β.

In contrast, hCG and IL-1β had no acute effect on cortisone reduction irrespective of the presence or absence of MA (Figure 6.5B). Co-treatment with MA inhibited 11KSR activity by approximately 50% ($p < 0.05$) irrespective of the presence or absence of hCG or IL-1β.

In the presence of AG, in human granulosa-lutein cells treated for 4 hours with hCG and IL-1 β , cortisol oxidation was increased to $132.2 \pm 9.4\%$ ($p>0.05$) and $128.8 \pm 4.5\%$ ($p<0.05$, Figure 6.6A) of control enzyme activity, respectively. Co-treatment with MA for 4 hours significantly decreased cortisol oxidation and prevented both hCG and IL-1 β from stimulating 11 β DH activity.

In the presence of AG, hCG and IL-1 β had no acute effect on cortisone reduction irrespective of the presence or absence of MA (Figure 6.6B).

6.3.3 Chronic effects of hCG and IL-1 β on 11 β HSD activities

In the absence of AG and in the absence of MA, hCG and IL-1 β tended to increase cortisol oxidation ($p>0.05$, Figure 6.7A). However, co-treatment with MA significantly inhibited the rate of cortisol oxidation in the presence of hCG and IL-1 β ($p<0.05$). As with acutely treated cells, in the absence of AG, hCG and IL-1 β had no significant effect on cortisone reduction ($p>0.05$, Figure 6.7B) irrespective of the presence of MA, which inhibited cortisone reduction by approximately 70% in control, hCG- and IL-1 β -treated cells.

In the presence of AG, hCG tended to increase cortisol oxidation by over 50% and IL-1 β significantly increased 11 β DH activity to $170.0 \pm 24.9\%$ ($p<0.05$, Figure 6.8A) of control 11 β DH activity. Co-treatment with MA abolished the stimulation of 11 β DH activity by both hCG and IL-1 β . As with acutely treated cells, both hCG and IL-1 β had no effect on 11KSR activity and co-treatment with MA inhibited cortisone reduction by approximately 50% (Figure 6.8B).

6.3.4 Concentration-dependent effects of hCG on PGE₂ and PGF_{2α} production

In the presence of AG, increasing concentrations of hCG had no effect on PGE₂ ($p>0.05$, Figure 6.9A), irrespective of the presence or absence of MA.

Treatment with increasing concentrations of hCG tended to increase PGF_{2α} production. The maximum effective concentration of 100ng/ml hCG increased PGF_{2α} production to $133.8 \pm 3.7\%$ of control values ($p>0.05$, Figure 6.9B). Treatment with MA abolished this stimulation.

6.3.5 Concentration-dependent effects of IL-1β on PGE₂ and PGF_{2α} production

IL-1β increased PGE₂ production in a concentration-dependent manner, with 10ng/ml IL-1β maximally increasing PGE₂ production to $154.9 \pm 21.0\%$ of control ($p<0.05$, Figure 6.10A). Co-treatment with MA abolished the stimulation of PGE₂ production by IL-1β.

IL-1β also tended to increase PGF_{2α} production ($p>0.05$, Figure 6.10B) and this stimulatory trend was abolished by co-incubation with MA.

6.3.6 Concentration-dependent effects of hCG on 11βHSD activities: effects of preferential-PGHS2 inhibitors

In the absence of MA, hCG exerted a concentration-dependent stimulation of 11βDH activity, with 100ng/ml hCG increasing 11βDH activity to $130.7 \pm 8.5\%$ of control enzyme activity ($p>0.05$, Figure 6.11A). In the presence of 100μM MA, these effects

of hCG on 11 β DH activity were abolished. Furthermore, MA inhibited 11 β DH activities by approximately 35 to 50% at each concentration of hCG. Treatment of human granulosa-lutein cells with hCG also tended to increase 11KSR activity ($p>0.05$, Figure 6.11B). Co-treatment with MA abolished this effect, decreasing 11KSR activity by 20 to 60% at each concentration of hCG.

In the absence of NS-398, increasing concentrations of hCG also tended to increase cortisol oxidation. Co-treatment with NS-398 inhibited the stimulation of 11 β DH activity by hCG (Figure 6.12).

6.3.7 Concentration-dependent effects of IL-1 β on 11 β HSD activities: effects of preferential PGHS2 inhibitors

Treatment of human granulosa-lutein cells with IL-1 β increased 11 β DH activity in a concentration-dependent manner. The maximum effective concentration of 10ng/ml IL-1 β increased 11 β DH activity to $140.8 \pm 5.5\%$ of control activity ($p<0.05$, Figure

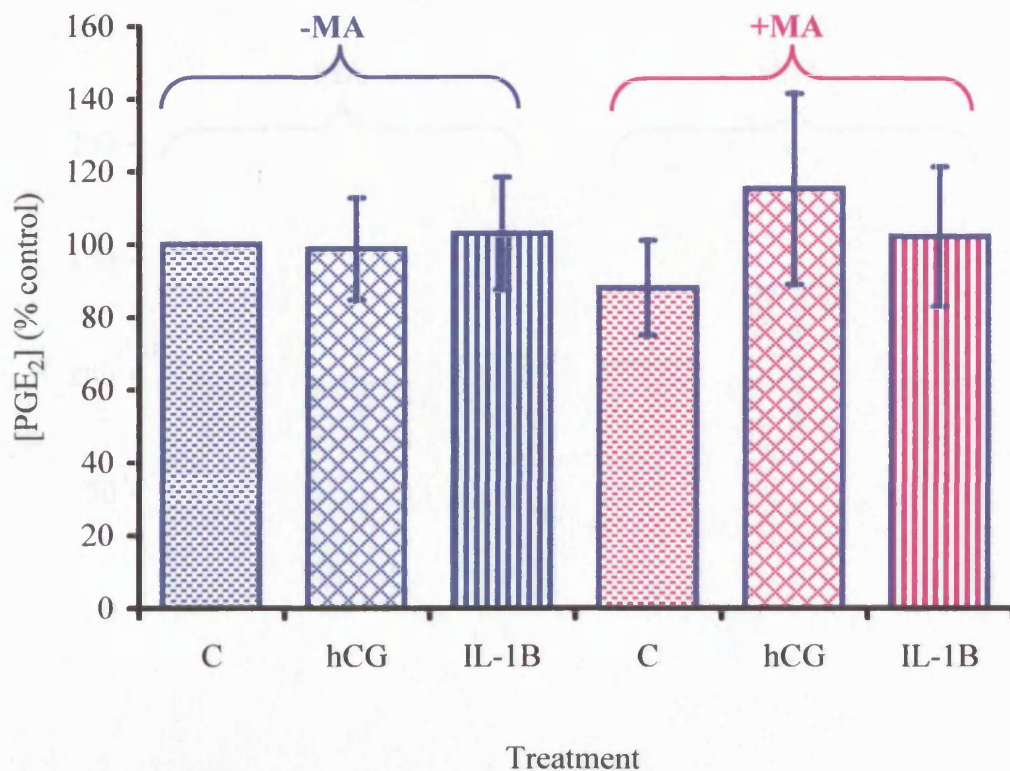


Figure 6.1A: Effects of hCG and IL-1 β on PGE₂ production over 4 hours in the absence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison.

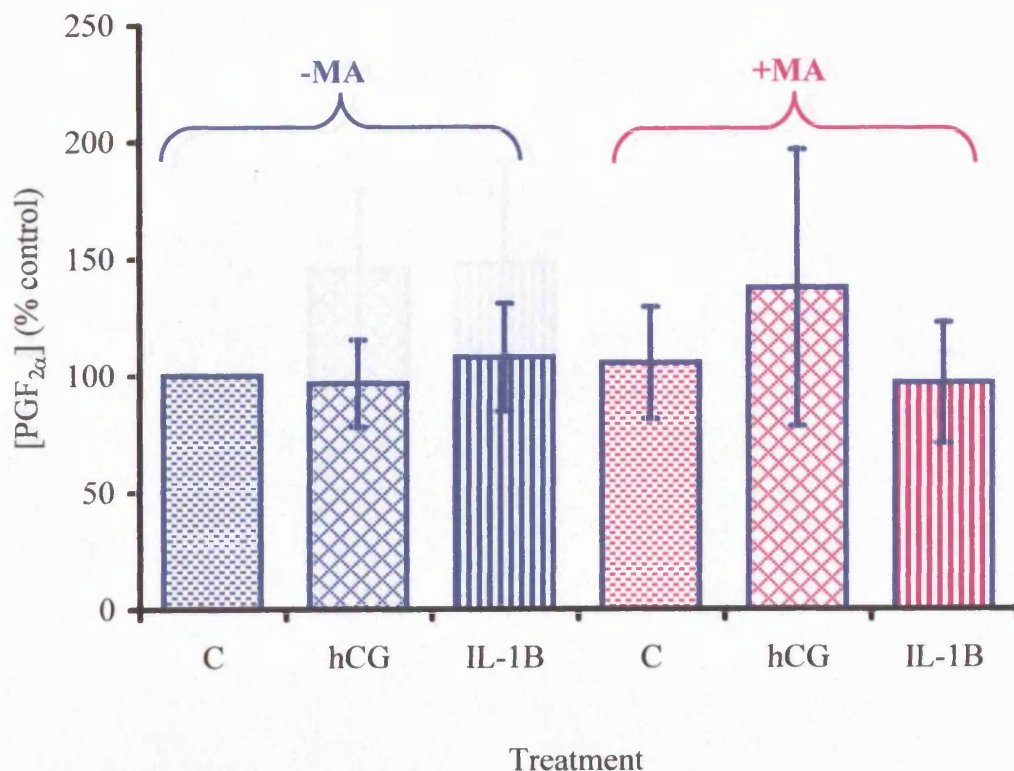


Figure 6.1B: Effects of hCG and IL-1 β on PGF_{2 α} production over 4 hours in the absence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm for 3 independent experiments ($p>0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

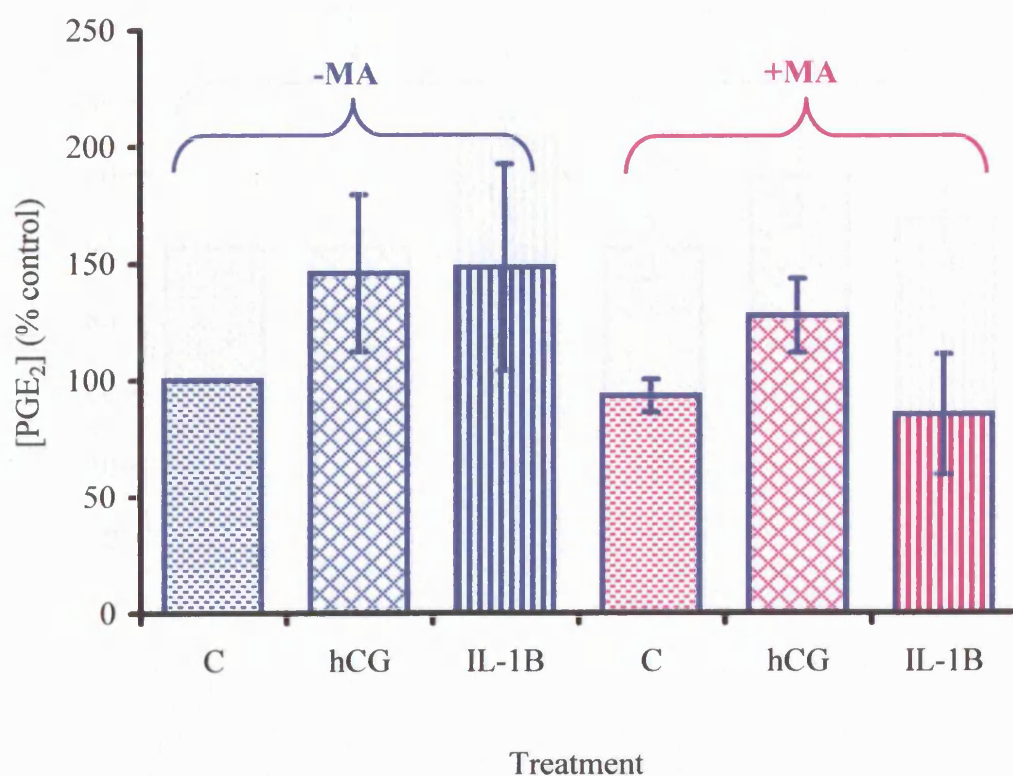


Figure 6.2A: Effects of hCG and IL-1 β on PGE₂ production over 4 hours in the presence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p>0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

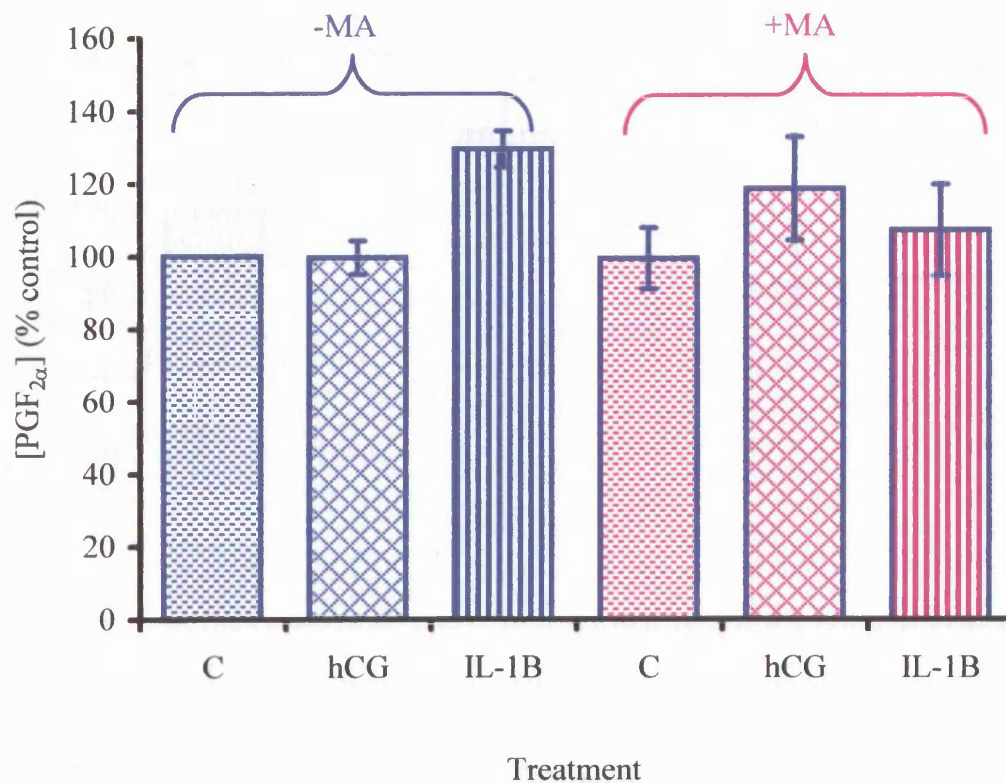


Figure 6.2B: Effects of hCG and IL-1 β on PGF_{2 α} production over 4 hours in the presence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

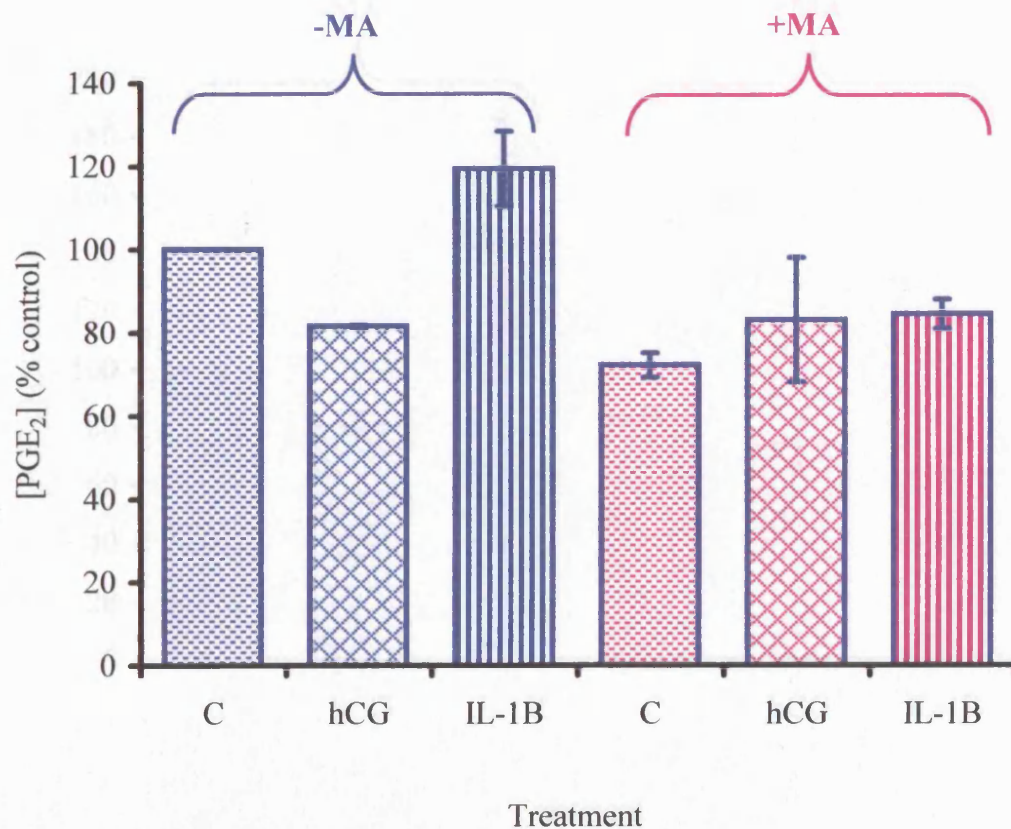


Figure 6.3A: Effects of hCG and IL-1 β on PGE₂ production over 24 hours in the absence of AG. Pink outlines to bars represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus control). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

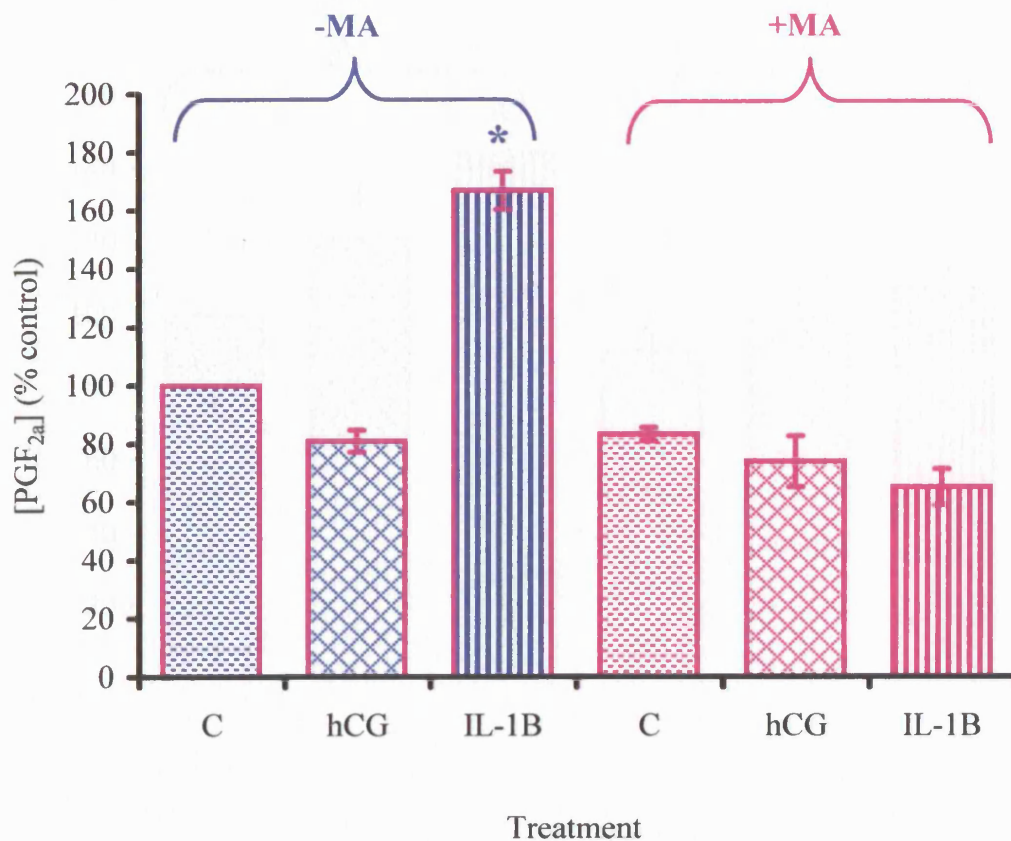


Figure 6.3B: Effects of hCG and IL-1 β on $PGF_{2\alpha}$ production over 24 hours in the absence of AG. Pink outlines to bars represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments (* $p < 0.05$ versus control). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

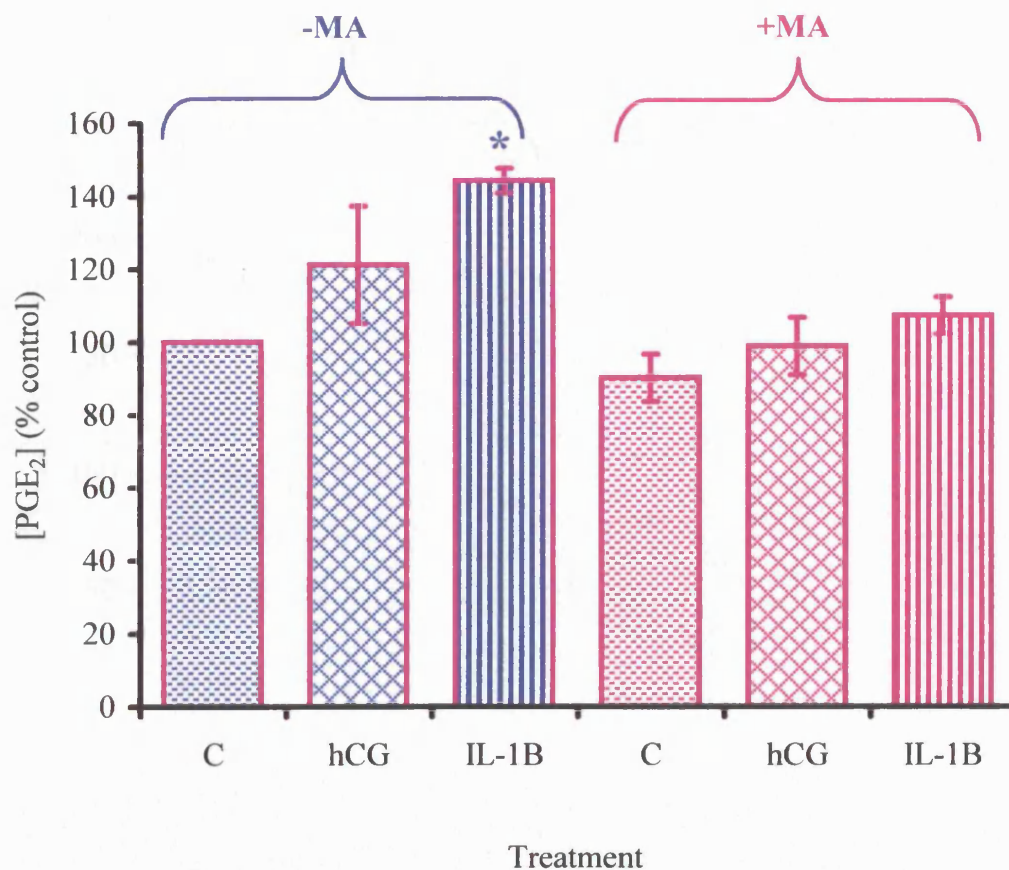


Figure 6.4A: Effects of hCG and IL-1 β on PGE₂ production over 24 hours in the presence of AG. Pink outlines to bars represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments (*p<0.05 versus control). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

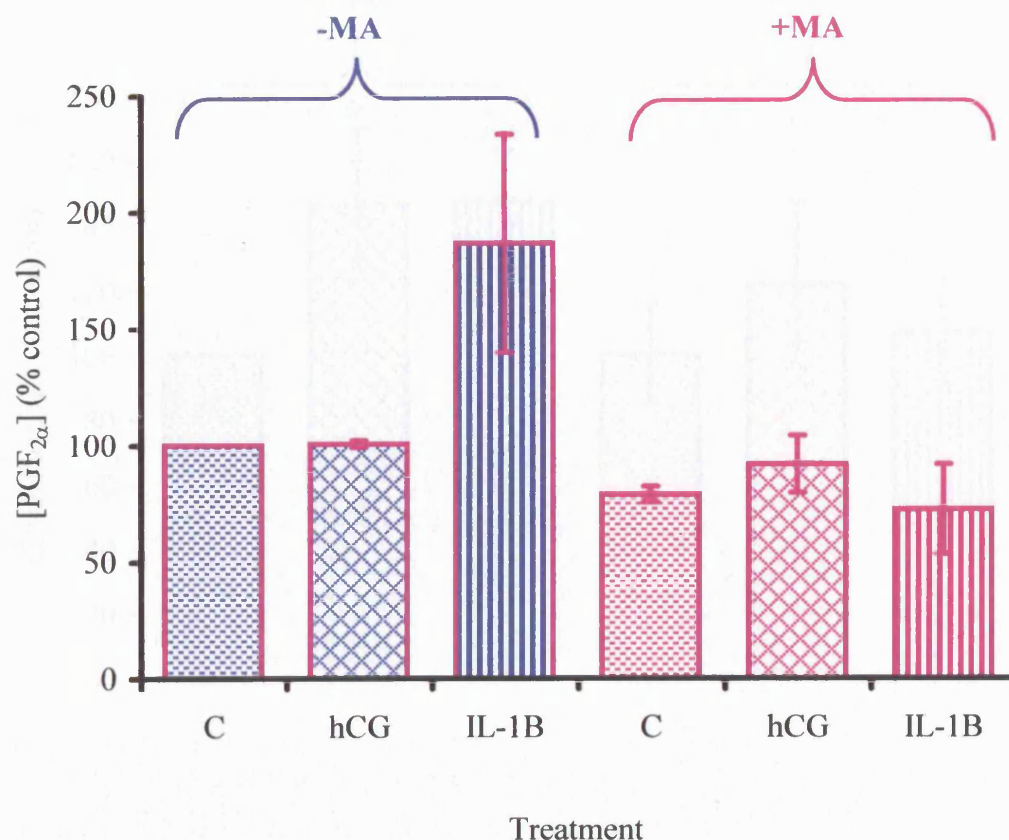


Figure 6.4B: Effects of hCG and IL-1 β on PGF_{2 α} production over 24 hours in the presence of AG. Pink outlines to bars represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

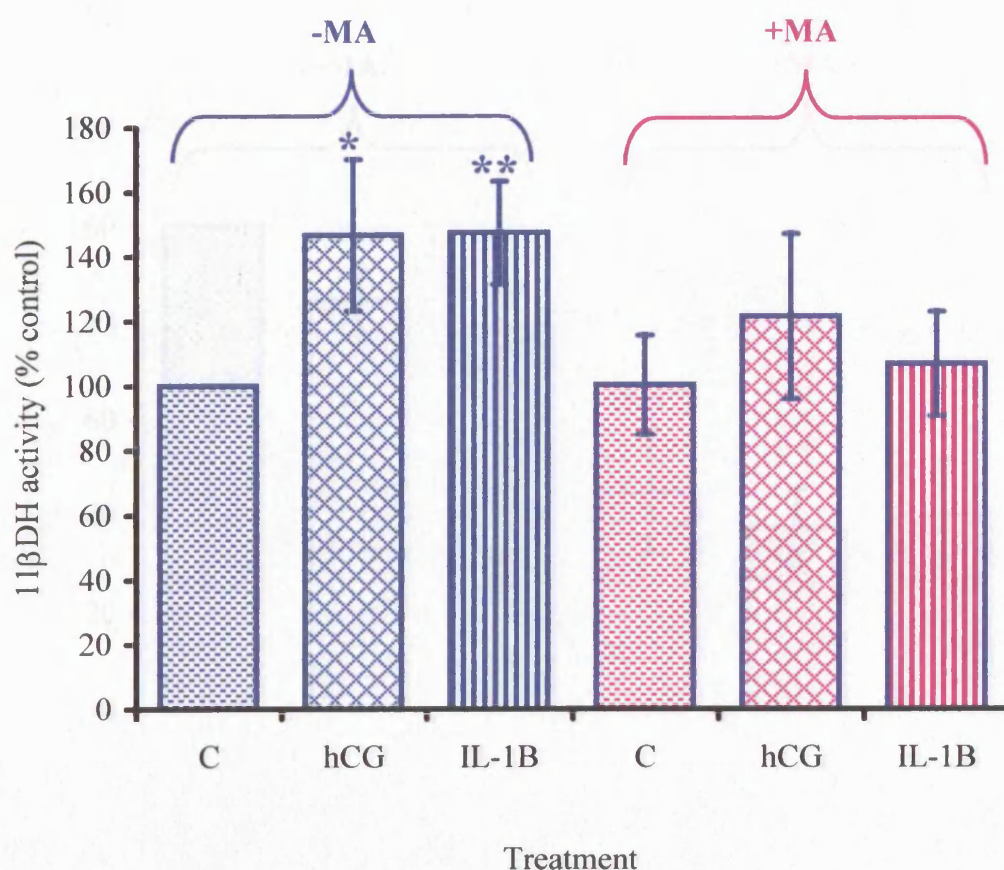


Figure 6.5A: Effects of hCG and IL-1 β on cortisol oxidation over 4 hours in the absence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 8 independent experiments (**p<0.01 versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

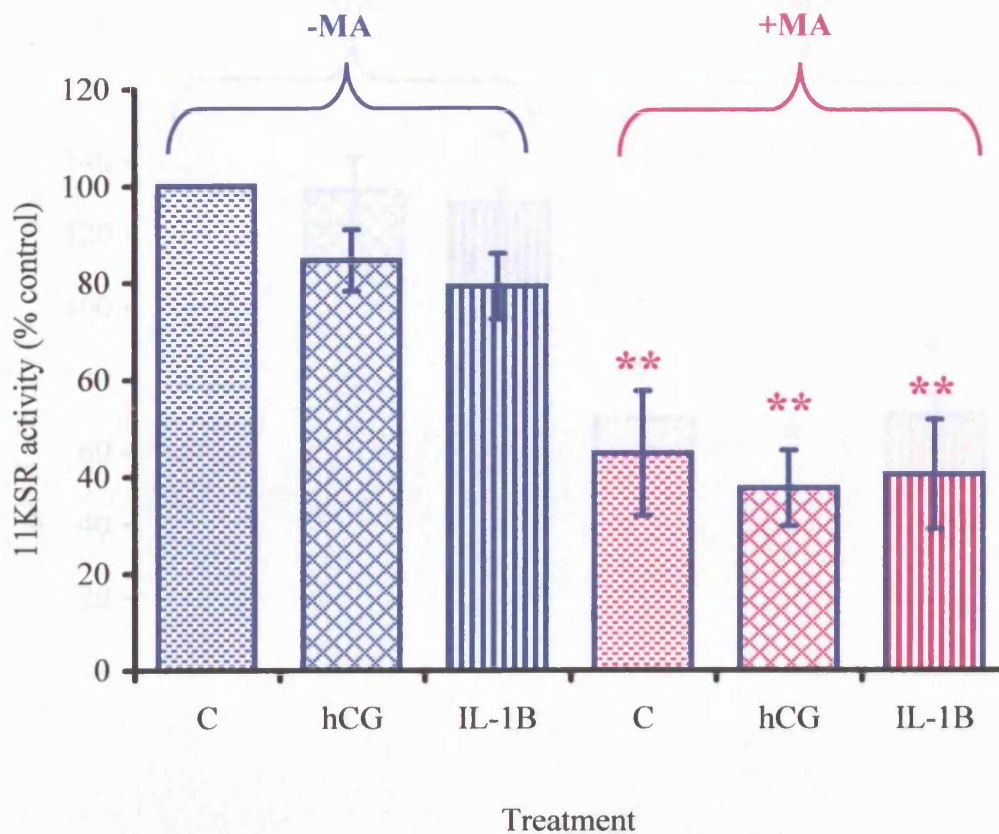


Figure 6.5B: Effects of hCG and IL-1 β on cortisone reduction over 4 hours in the absence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 5 independent experiments ($p > 0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

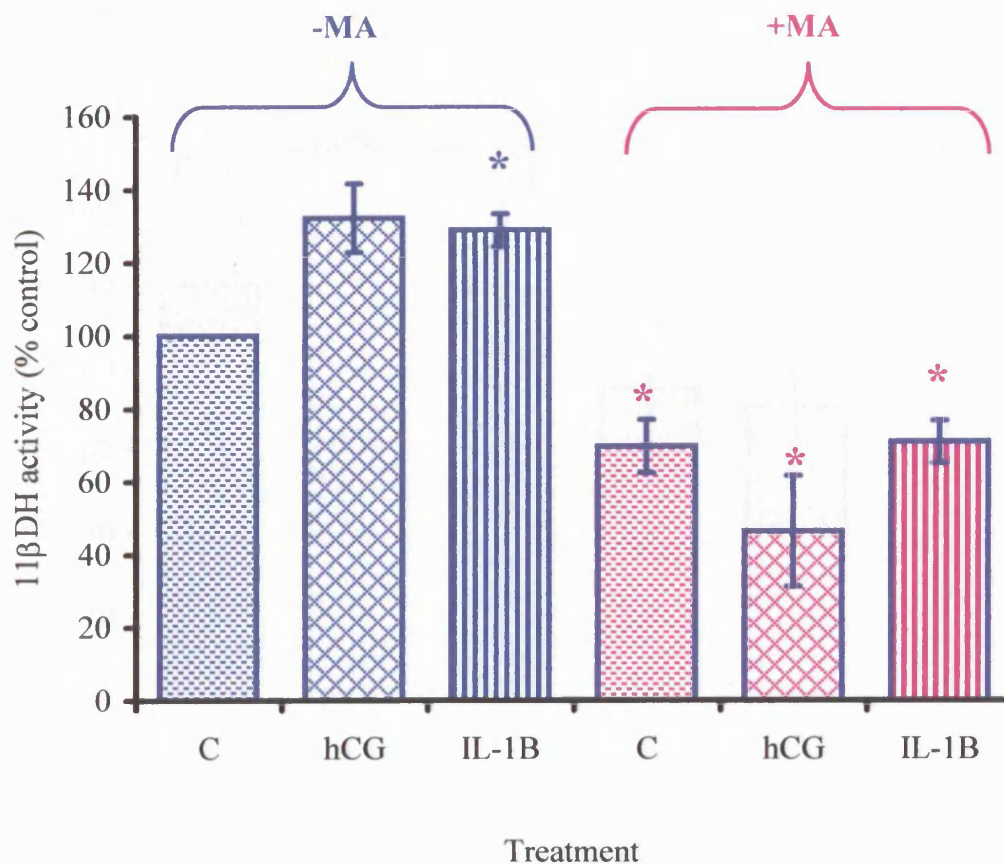


Figure 6.6A: Effects of hCG and IL-1 β on cortisol oxidation over 4 hours in the presence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 8 independent experiments (** p <0.01 versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

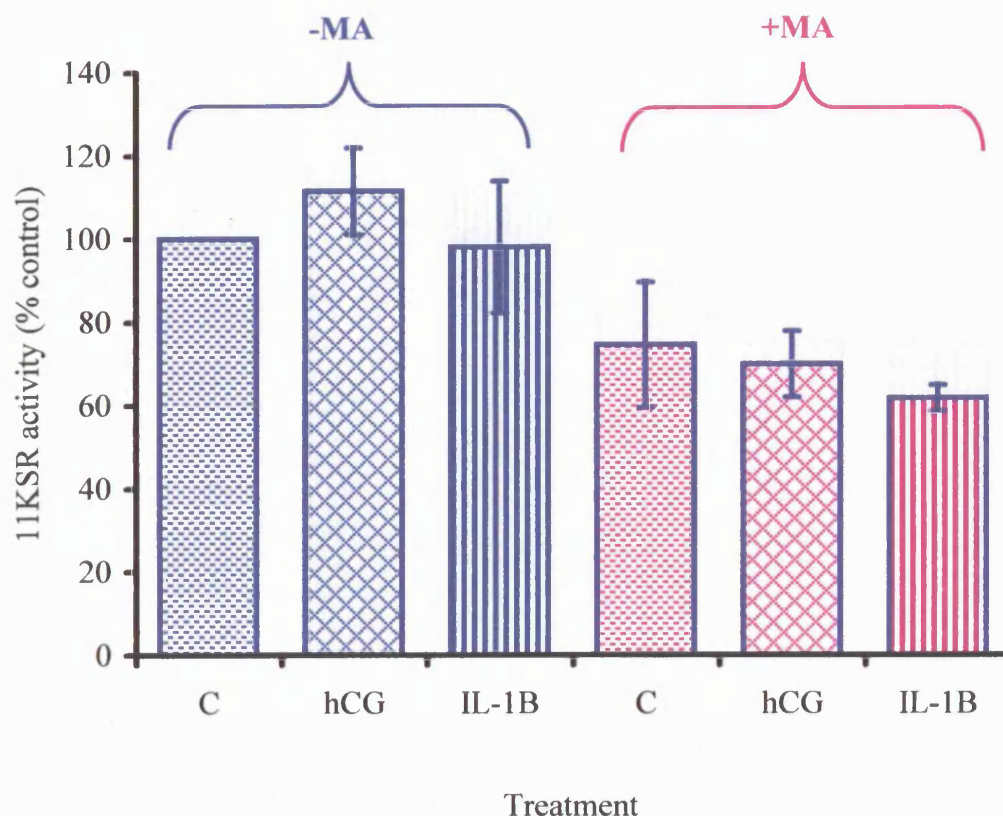


Figure 6.6B: Effects of hCG and IL-1 β on cortisone reduction over 4 hours in the presence of AG. Blue outlines represent 4 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p>0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

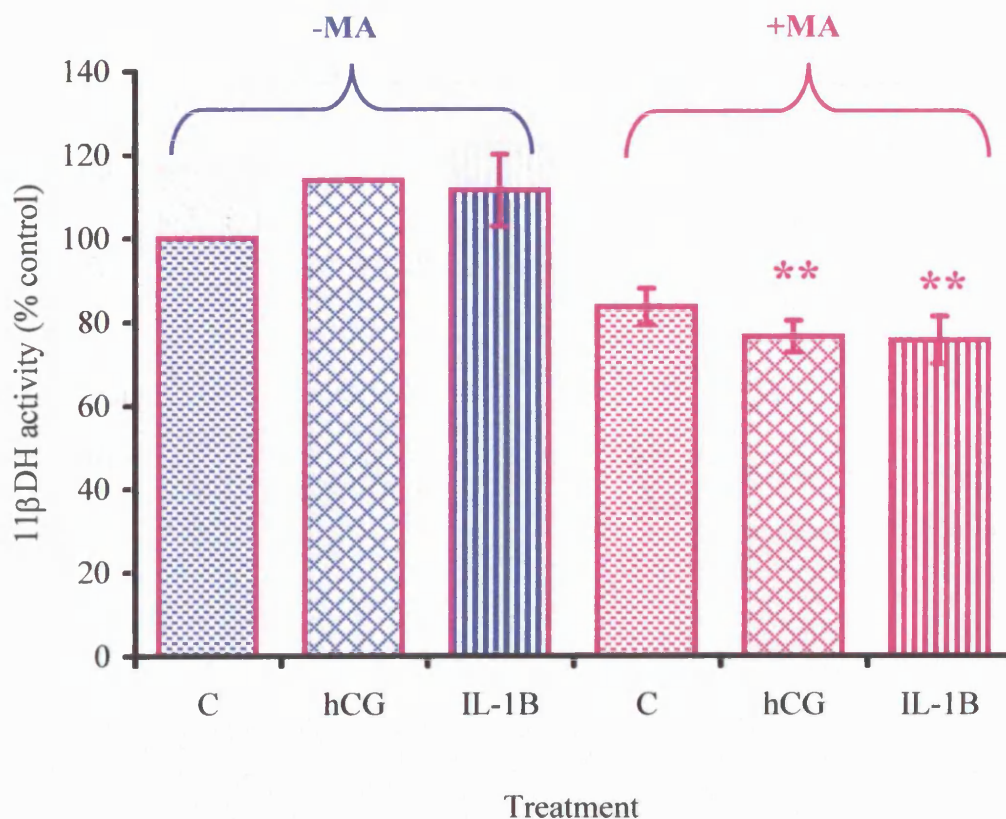


Figure 6.7A: Effects of hCG and IL-1 β on cortisol oxidation over 24 hours in the presence of AG. Pink outlines represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments (*p<0.05 versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

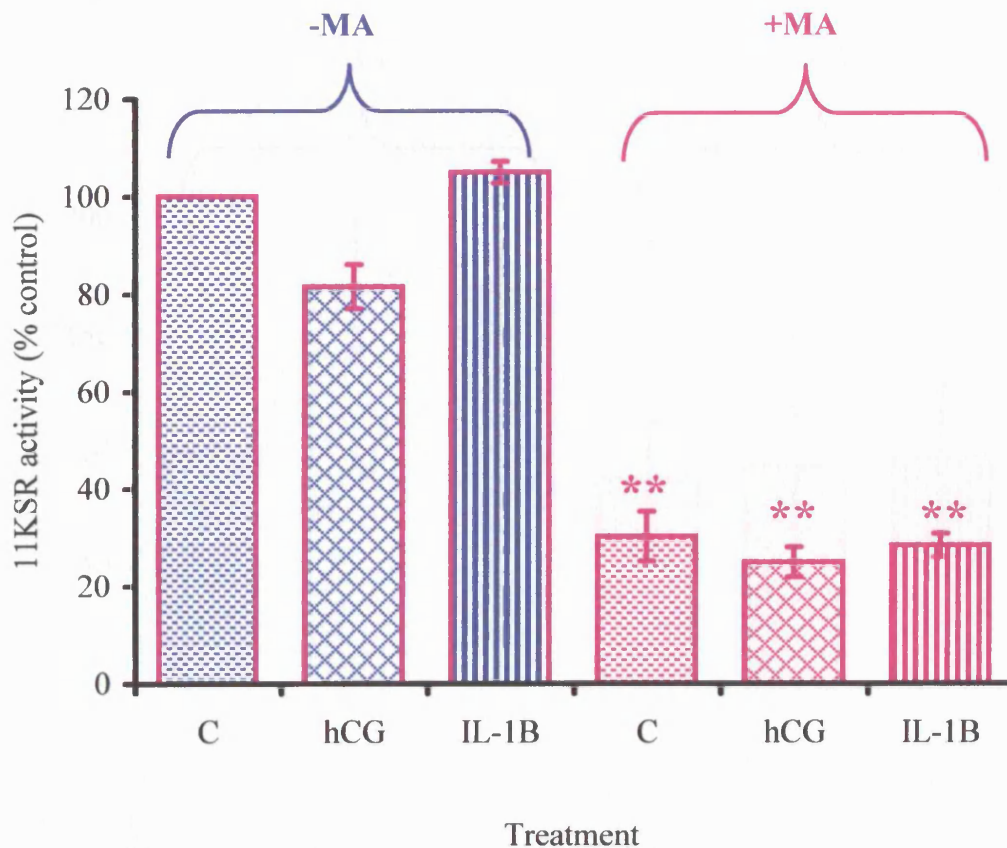


Figure 6.7B: Effects of hCG and IL-1 β on cortisone reduction over 24 hours in the presence of AG. Pink outlines represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

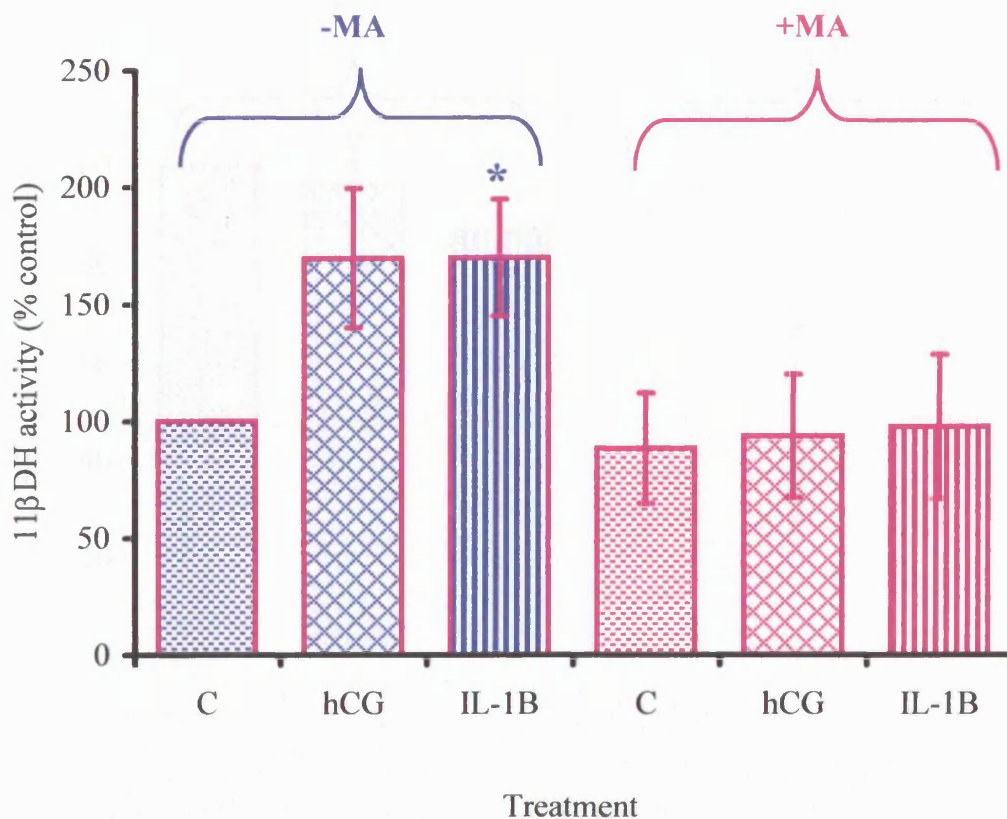


Figure 6.8A: Effects of hCG and IL-1 β on cortisol oxidation over 24 hours in the presence of AG. Pink outlines represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments (* p <0.05 versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

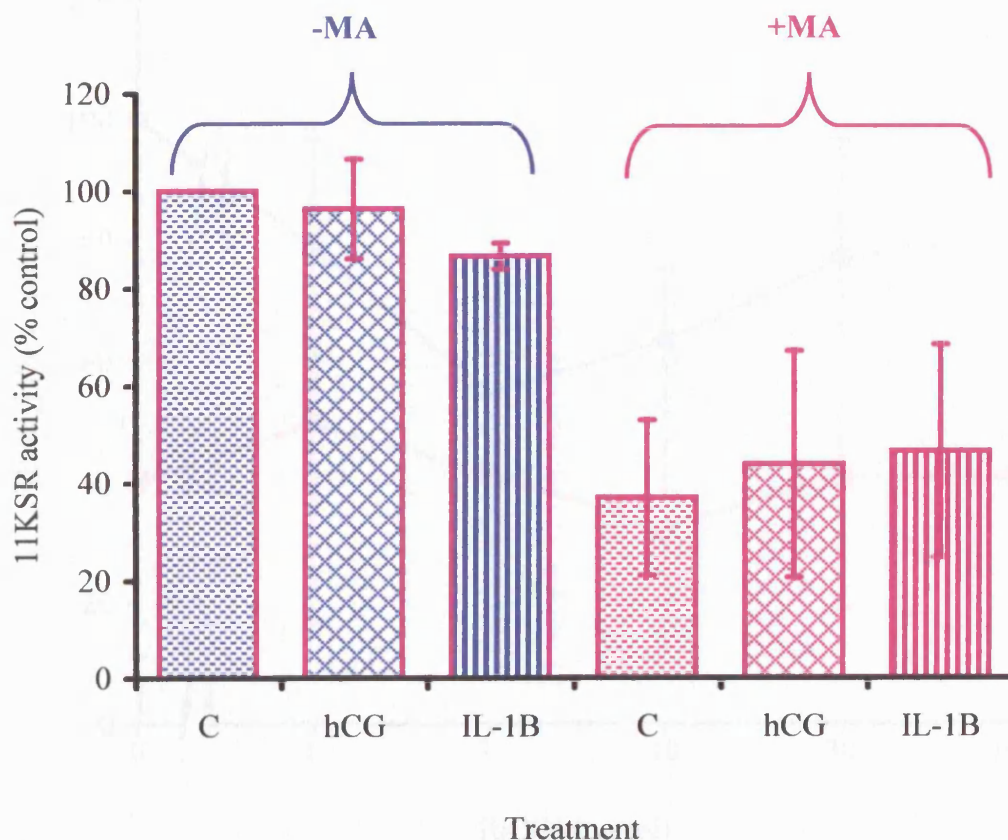


Figure 6.8B: Effects of hCG and IL-1 β on cortisone reduction over 24 hours in the presence of AG. Pink outlines represent 24 hour time point, Blue fill to graphs and brackets represent conditions minus MA, Pink fill of graphs and brackets represent conditions plus MA. C =control, hCG=100ng/ml, IL-1B=10ng/ml. Each data point represents the mean \pm SEM for 3 independent experiments ($p>0.05$ versus control minus MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison

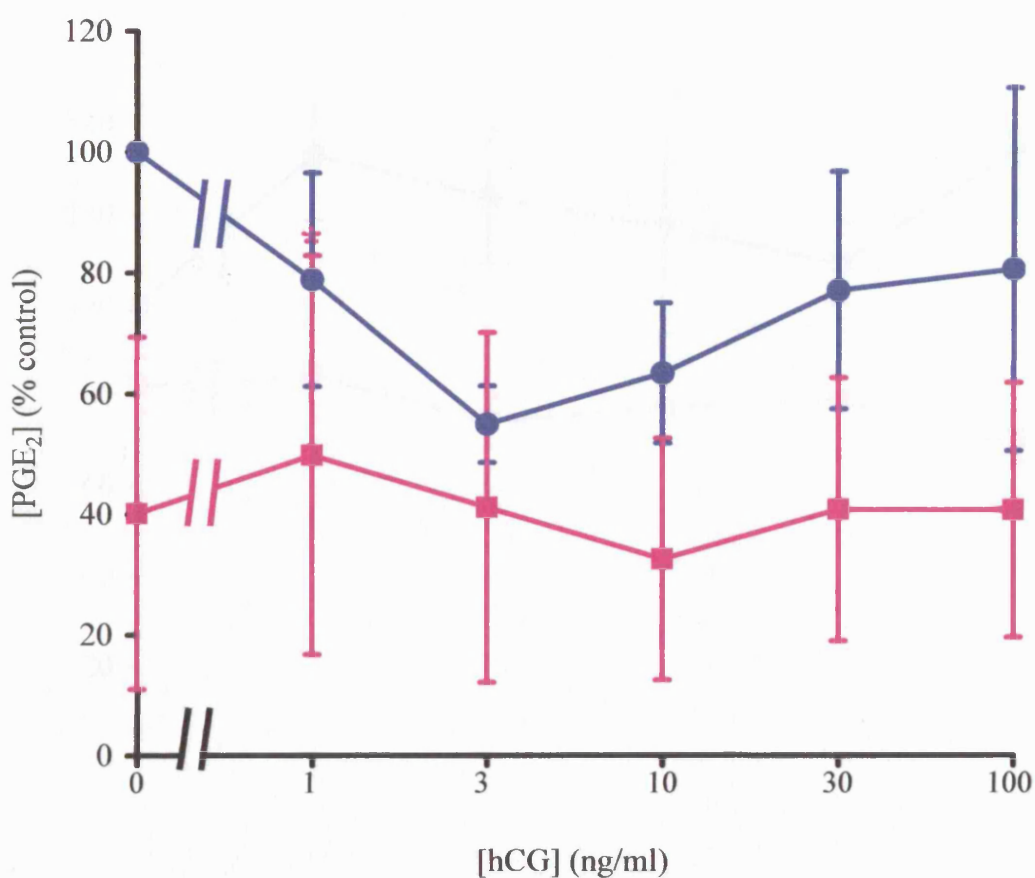


Figure 6.9A: Concentration dependent effects of hCG on PGE₂ over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 5 independent experiments ($p > 0.05$ versus 0 μ M MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

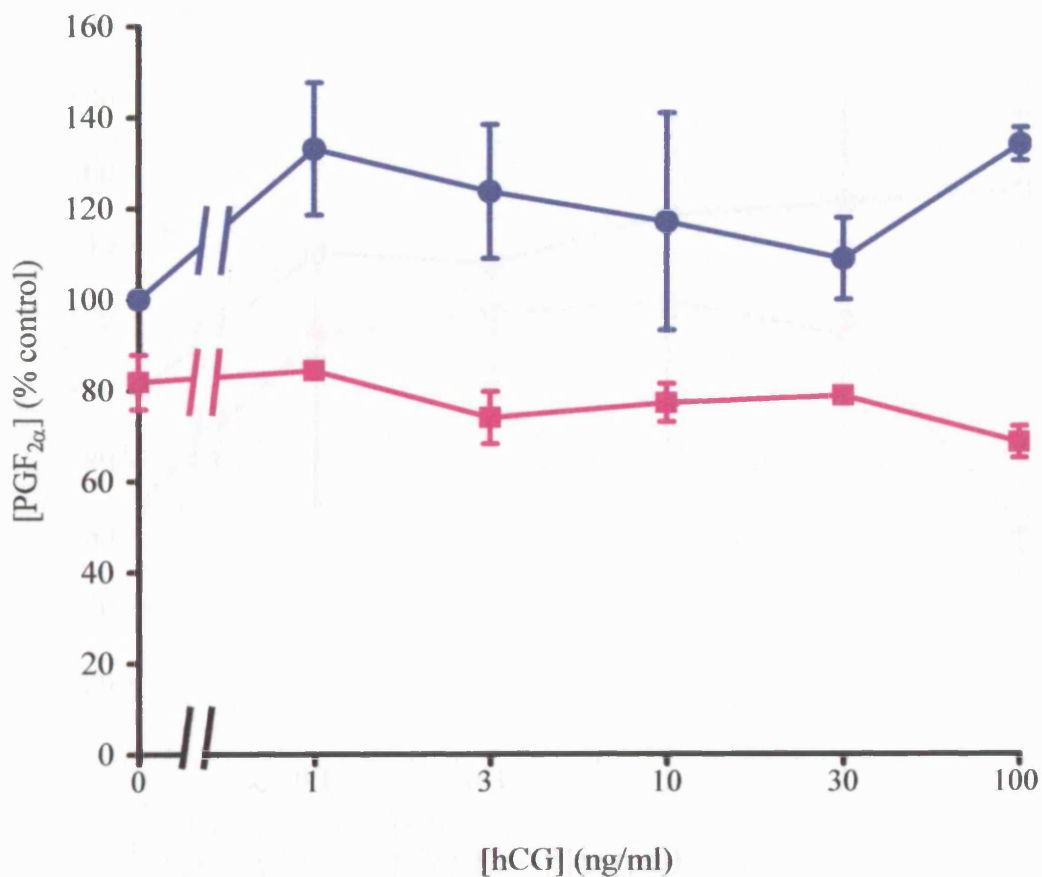


Figure 6.9B: Concentration dependent effects of hCG on PGF_{2α} over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 4 independent experiments ($p > 0.05$ versus 0 μ M MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

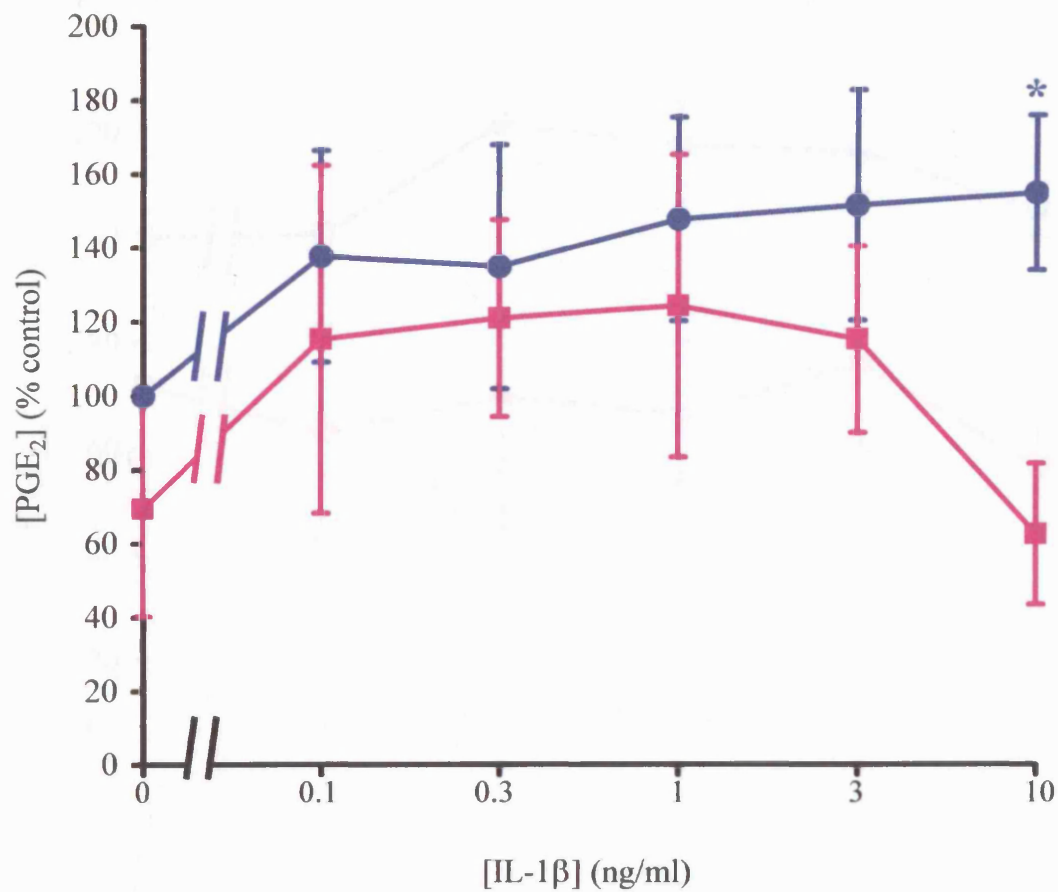


Figure 6.10A: Concentration dependent effects of IL-1 β on PGE₂ over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 5 independent experiments ($p > 0.05$ versus 0 μ M MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

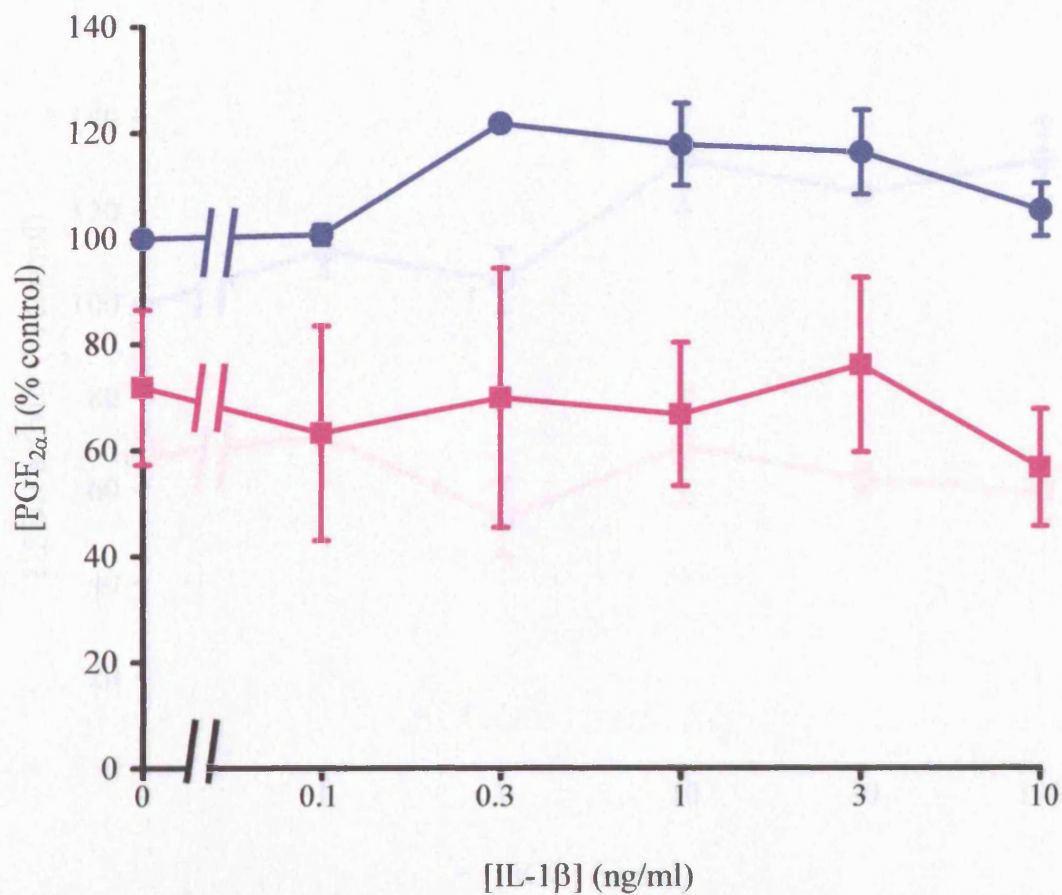


Figure 6.10B: Concentration dependent effects of IL-1 β on PGF_{2 α} over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 4 independent experiments ($p > 0.05$ versus 0 μ M MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

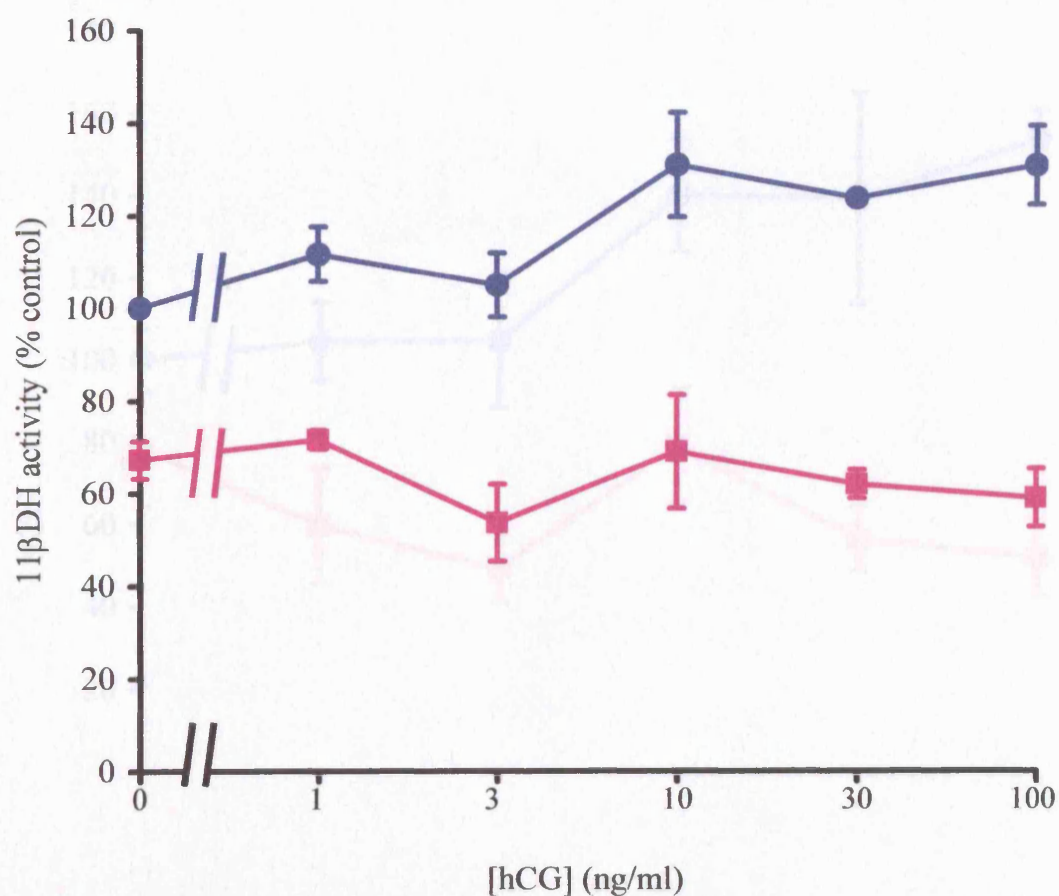


Figure 6.11A: Concentration dependent effects of hCG on cortisol oxidation over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus $0 \mu\text{M}$ MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

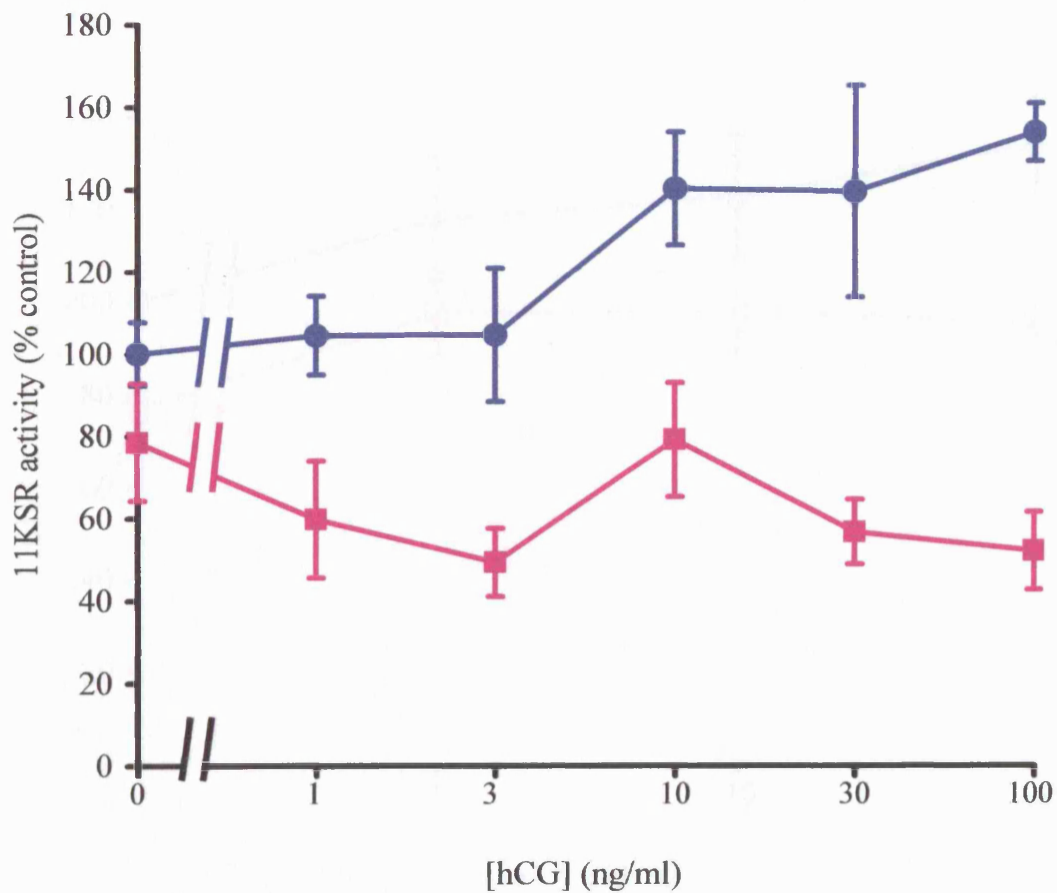


Figure 6.11B: Concentration dependent effects of hCG on cortisone reduction over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus $0\mu\text{M}$ MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

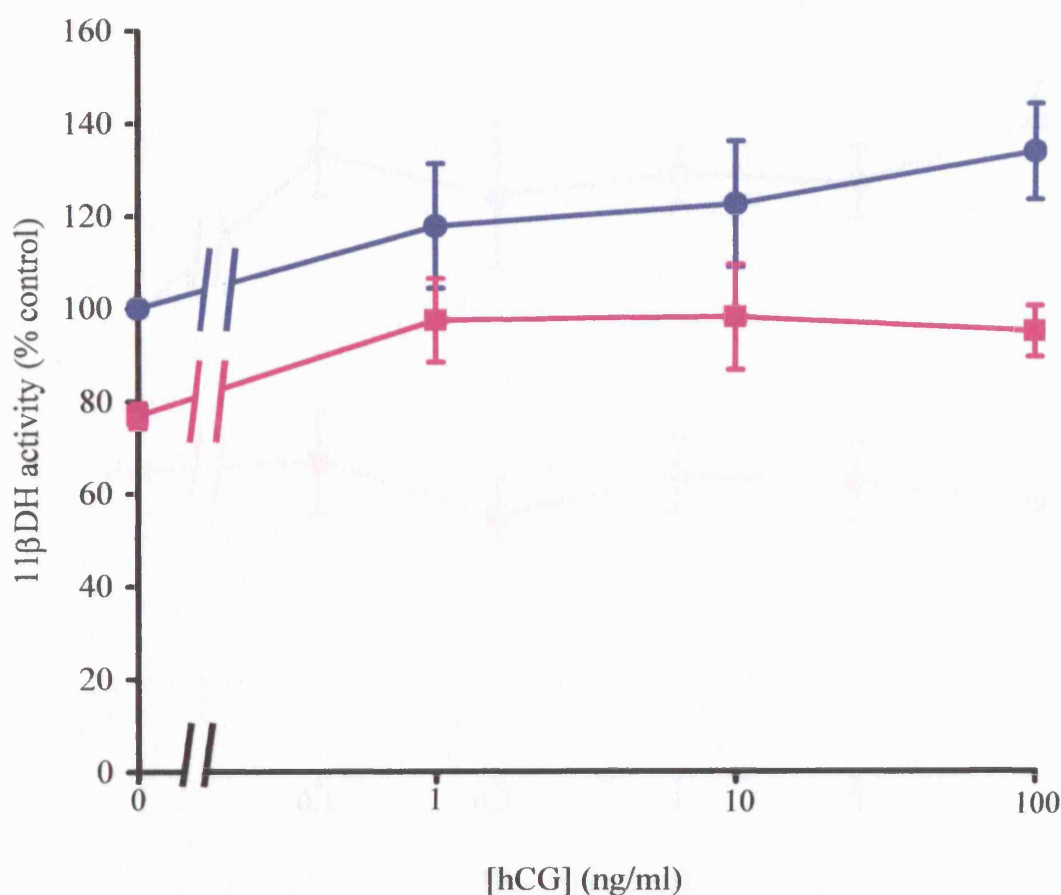


Figure 6.12: Concentration dependent effects of hCG on cortisol oxidation over 24 hours in the presence of AG. Blue lines represent conditions minus NS-398, Pink lines represent conditions plus NS-398. Experiments conducted in presence of vehicle (1% (v/v) DMSO). Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus $0 \mu\text{M}$ NS-398). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus NS-398

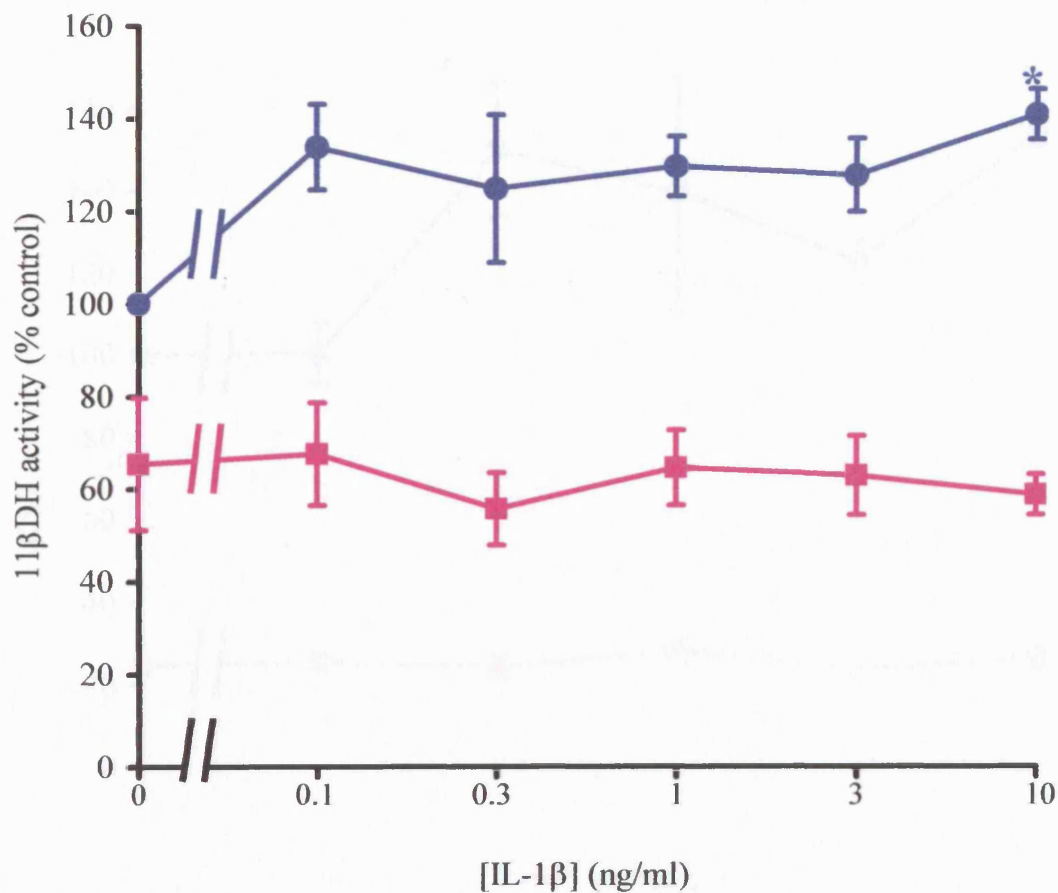


Figure 6.13A: Concentration dependent effects of IL-1 β on cortisol oxidation over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 3 independent experiments (* $p < 0.05$ versus 0 μ M MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

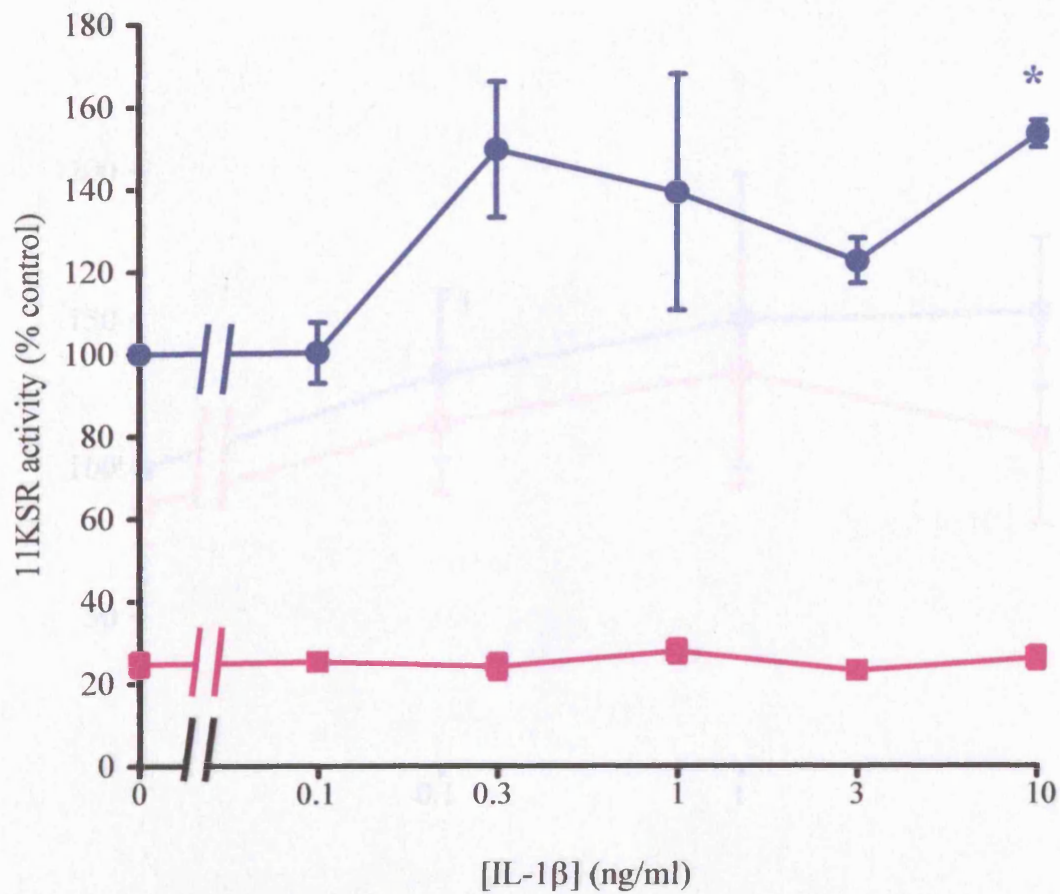


Figure 6.13B: Concentration dependent effects of IL-1 β on cortisone reduction over 24 hours in the presence of AG. Blue lines represent conditions minus MA, Pink lines represent conditions plus MA. Each data point represents the mean \pm SEM for 3 independent experiments (*p<0.05 versus 0 μ M MA). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus MA

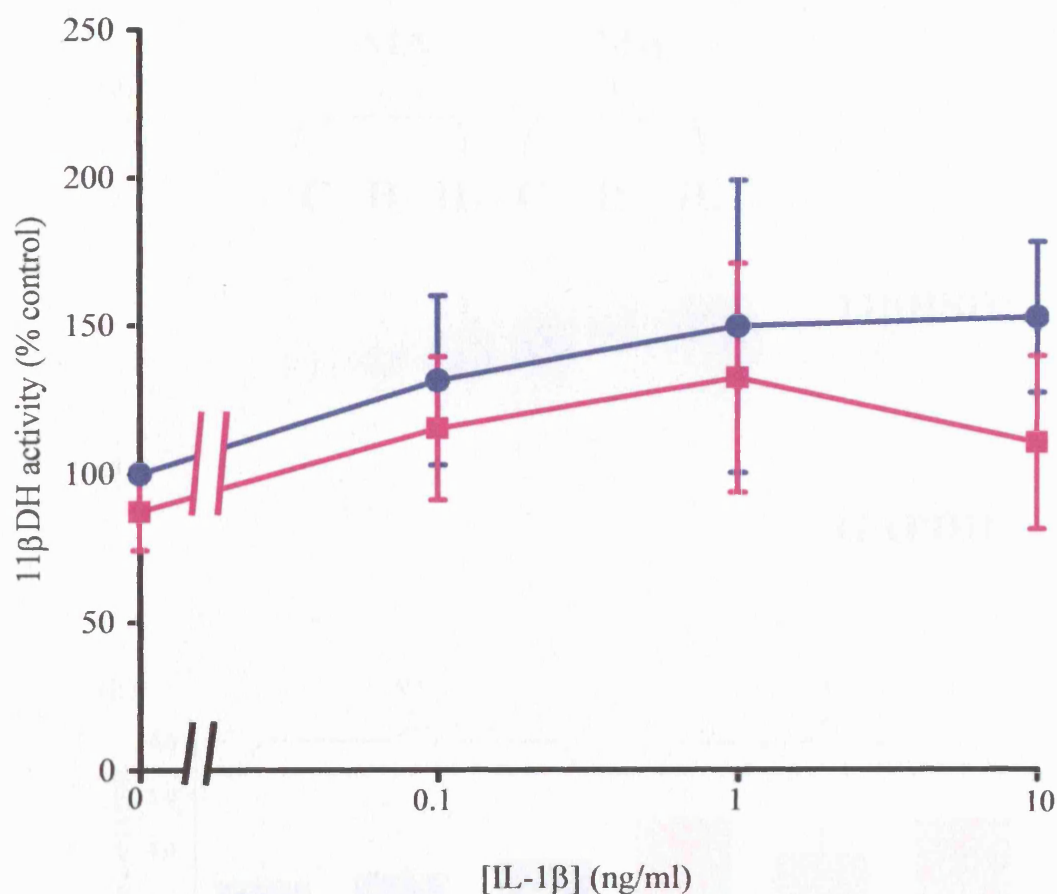


Figure 6.14: Concentration dependent effects of IL-1 β on cortisol oxidation over 24 hours in the presence of AG. Blue lines represent conditions minus NS-398, Pink lines represent conditions plus NS-398. Experiments conducted in presence of vehicle (1% (v/v) DMSO). Each data point represents the mean \pm SEM for 3 independent experiments ($p > 0.05$ versus 0 μ M NS-398). Data were analysed by one-way ANOVA with repeated measures followed by Dunnett's multiple comparison versus control minus NS-398

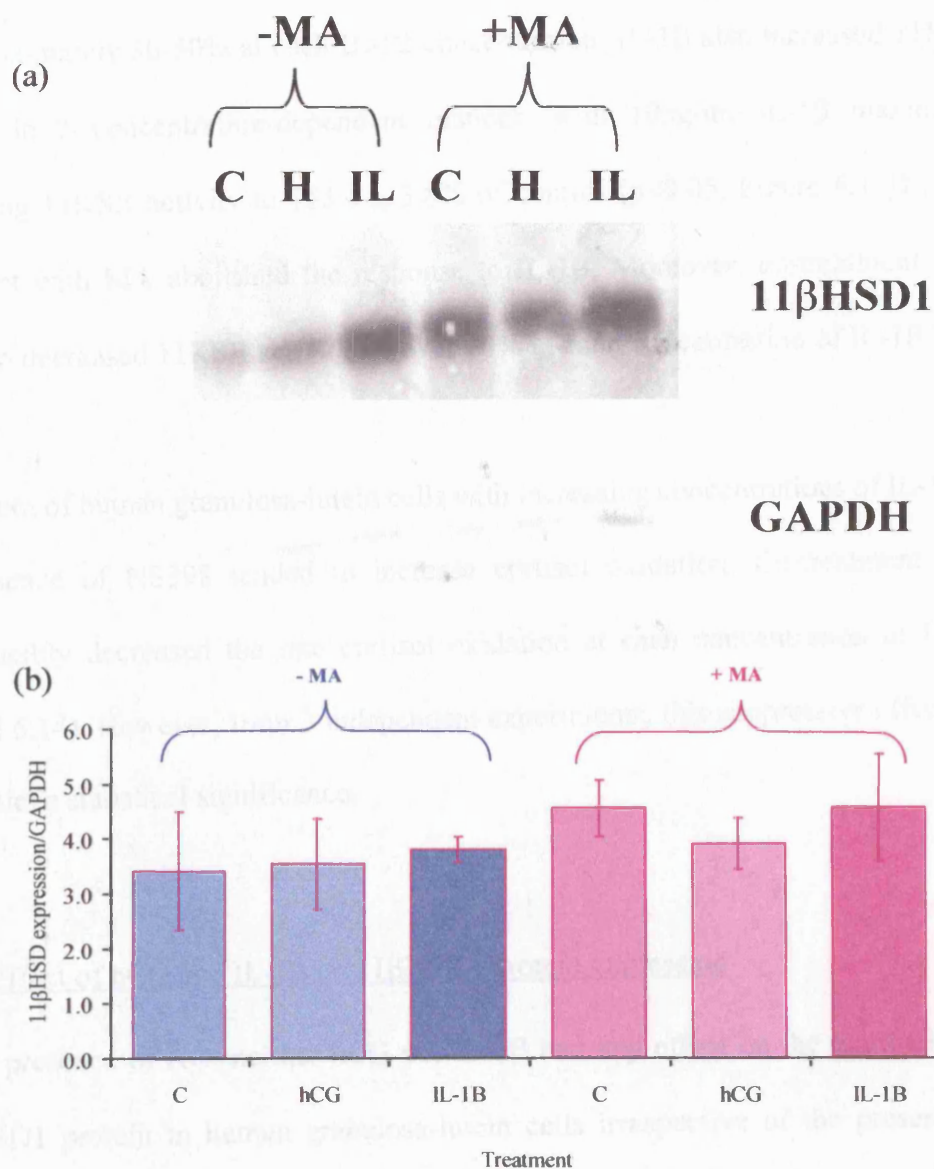


Figure 6.15: Effect of hCG and IL-1 β on 11 β HSD1 protein expression of 11 β HSD1 standardised to GAPDH protein expression in the presence of AG. (a) Western blot showing effect of hCG and IL-1 β on 11 β HSD1 protein expression relative to GAPDH on day 3 of culture in human granulosa lutein cells. C represents control conditions, hCG represents treatment with 100ng/ml hCG, and 10ng/ml IL-1 β (b) Densitometric representation of effect of hCG and IL-1 β on 11 β HSD1 protein expression relative to GAPDH. The blue bars represent experiments carried out in the absence of 100 μ M MA. The pink bars represents experiments carried out in the presence of 100 μ M MA. Data represents the mean \pm SEM for 3 independent experiments $p > 0.05$. Data analysed by one-way ANOVA with Dunnett's multiple comparison for each treatment

6.13A). Co-treatment with MA abolished this stimulation decreasing 11 β DH activity by approximately 30-50% at each IL-1 β concentration. IL-1 β also increased 11KSR activity in a concentration-dependent manner, with 10ng/ml IL-1 β maximally increasing 11KSR activity to $153.4 \pm 3.8\%$ of control ($p < 0.05$, Figure 6.13B). Co-treatment with MA abolished the response to IL-1 β . Moreover, co-treatment with MA also decreased 11KSR activity by over 70% at each concentration of IL-1 β .

Treatment of human granulosa-lutein cells with increasing concentrations of IL-1 β in the absence of NS398 tended to increase cortisol oxidation. Co-treatment with reproducibly decreased the rate cortisol oxidation at each concentration of IL-1 β (Figure 6.14). However, from 3 independent experiments, this suppressive effect did not achieve statistical significance.

6.3.8 Effect of hCG and IL-1 β on 11 β HSD1 protein expression

In the presence of AG, neither hCG nor IL-1 β had any effect on the expression of 11 β HSD1 protein in human granulosa-lutein cells irrespective of the presence or absence of MA ($p > 0.05$, Figure 6.15).

6.4 Discussion

This study has shown that hCG and IL-1 β can increase 11 β HSD activities in a concentration-dependent manner without changes in 11 β HSD1 protein expression. Co-treatment with 2 preferential inhibitors of PGHS2, MA and NS-398, attenuated the stimulation of 11 β HSD activities by hCG and IL-1 β . hCG and IL-1 β were

confirmed to increase PGE₂ and PGF_{2α} production by human granulosa-lutein cells, and co-treatment with MA was shown to block this stimulation of prostaglandin production.

A difference in the ability of hCG and IL-1β to affect cortisol oxidation has been observed. IL-1β was more effective at stimulating cortisol oxidation than hCG, with IL-1β significantly stimulating cortisol oxidation in all experiments. In the absence of AG, the differences in potencies of hCG and IL-1β could be due to hCG stimulating progesterone production by human granulosa-lutein cells. Studies in Chapter 4 indicated that progesterone production can inhibit basal 11βHSD activities. Therefore this stimulation of progesterone production by hCG could be attenuating any stimulatory effects of hCG on cortisol oxidation. However, the addition of AG (to inhibit progesterone production) did not alter the lack of effect of hCG on 11βDH activities. On the basis of these data alone, it is not possible to exclude a role for progesterone in limiting the 11βHSD response to hCG since 100μM AG is only partially successful in suppressing progesterone synthesis.

It is noteworthy that in preliminary single concentration experiments, hCG and IL-1β did not stimulate cortisone reduction in either acutely or chronically treated human granulosa-lutein cells. However, in concentration-dependent experiments carried out in the presence of AG, hCG tended to increase cortisone reduction, and IL-1β significantly increased cortisone reduction. On balance, the data reported in this Chapter indicate that hCG and IL-1β exert more reproducible, consistent stimulation

of cortisol oxidation than on cortisone reduction in human granulosa-lutein cells treated for either 4 or 24 hours.

A difference in potency was observed between MA and NS-398 in attenuating hormone-stimulated 11 β HSD activities was observed. In the presence of increasing concentrations of either hCG or IL-1 β , MA inhibited 11 β HSD activities by approximately 30-40% in comparison to around 20% inhibition by NS-398. These findings were consistent the data reported in Chapter 5. Moreover, under basal conditions, MA inhibited prostaglandin production to a greater extent than NS-398. This reflects the different properties of the 2 compounds reviewed in Chapter 5. In brief the different properties of the inhibitors could reflect differences in solubility. Other possible explanations for the differences include the selectivity of MA and NS-398 for inhibiting PGHS-2 and not PGHS-1, or indeed for inhibiting other pathways of AA metabolism. These hypotheses have been reviewed in more detail in section 5.4.

Small increases in PGE₂ and PGF_{2 α} were observed in cells incubated with hCG or IL-1 β . These data are consistent with several reports that have documented the stimulation of PGE₂ and PGF_{2 α} by IL-1 β in the rat [Carnovale *et al.*, 2001], bovine [Del Vecchio and Sutherland 1997] and primate ovary [Young *et al.*, 1997]. hCG has been documented previously to increase PGHS2 expression and PGE₂ production in mucosal cells from human fallopian tubes [Han *et al.*, 1996]

IL-1 β could be increasing prostaglandin production through a number of mechanisms. Studies by Brown *et al.* [1998] in intact foetal membranes have shown that IL-1 β can increase PGE₂ production by increasing PGHS2 mRNA levels and inducing the translocation of cytoplasmic phospholipase A₂ (cPLA₂) from the cytosol to the membrane. In bovine luteal cells, the addition of a PLA₂ inhibitor and an inhibitor of protein kinase C (PKC) was shown to abolish IL-1 β stimulated PGF_{2 α} production [Townson and Pate 1994]. Studies using the phorbol ester 4- β -phorbol 12-myristate 13-acetate (PMA), to activate PKC, have shown that PMA is able to activate PGHS2 gene transcription. This is thought to be via the kinase pathways leading to the activation of activating protein-1 (AP-1) and activating transcription factor (ATF), and through the induction of PGHS2 gene transcription through binding to the CRE/ATF binding sites of the PGHS2 gene [Catley *et al.*, 2003]. Other studies have implicated nuclear factor-kappaB (NF- κ B) as a transcription factor mediating the stimulation of prostaglandin synthesis by IL-1 β through up-regulating PGHS2 mRNA expression [Catley *et al.*, 2003]. NF- κ B transcriptional binding sites have been located on the PGHS2 gene, and the binding of NF- κ B to these sites are thought to activate the PGHS2 promoter to induce gene transcription [Catley *et al.*, 2003].

Studies have revealed that the mechanism by which hCG up-regulates PGHS2 mRNA levels is not through increasing the transcription of the gene, but through increasing the stability of the transcript [Han *et al.*, 1996a: 1996b]. In addition to this, in rat ovarian cells, soluble PLA₂ expression was shown to be hCG dependent [Ben-Shlomo *et al.*, 1997]. Other studies have revealed that ovarian PLA₂ activity

was stimulated by hCG administration in rats [Bonney and Wilson 1993]. These data together reveal that hCG/LH can increase prostaglandin production through increasing the expression and activity of PLA₂ and through increasing the stability of PGHS2 mRNA. Indirect effects of hCG on stimulating prostaglandin production have also been documented, with NS-398 and indomethacin administration shown to reduce the number of ovulations in the rat [Mikuni *et al.*, 1998].

The data presented in this Chapter could be interpreted to suggest that prostaglandins play a mediatory role in the stimulation of 11 β HSD activities by hCG and IL-1 β . There are a number of studies that have documented the role of prostaglandins in increasing the activity and expression of 11 β HSD1 in different tissue types. In human chorionic trophoblast cells, studies by Alfaidy *et al.* [2001] have documented that PGF_{2 α} can increase 11 β HSD1 activity. This stimulation was speculated to occur via FP receptor activation and subsequent mobilisation of calcium stores. This is thought to activate calcium dependent protein kinase (PKC), which has been shown to increase the phosphorylation of 11 β HSD1, speculated to account for the increase in 11 β HSD1 activity. Consensus sites for PKC phosphorylation are present in the primary sequence of 11 β HSD1, such that this pathway could mediate the effects of prostaglandins on regulating the activity (but not expression) of 11 β HSD1 in human granulosa-lutein cells. However, subsequent studies in this thesis question the ability of PKC to access 11 β HSD1 (see Chapter 7).

Other mechanisms by which prostaglandins could be regulating 11 β HSD1 activity are via peroxisome proliferator-gamma receptors (PPARs) subtype. Prostaglandins

are naturally occurring ligands of PPARs [Helliwell *et al.*, 2004]. 15deoxy-PGJ₂, the suggested ligand for PPAR γ has been shown to increase 11 β HSD1 activity in omental and subcutaneous adipocyte stromal cells. Moreover, inhibitors of PPAR γ have been shown to inhibit 11 β HSD1 activity, and Tomlinson *et al.* [2001] have speculated that the effects of PGJ₂ on increasing 11 β HSD1 activity could be mediated by PPAR γ . However, as PPARs are nuclear receptors, they would be expected to influence the activity of 11 β HSD through changing 11 β HSD expression. Studies reported in chapters 5 and 6 of this thesis do not find any changes of protein expression, but do not exclude changes in expression of 11 β HSD1 mRNA.

One possible mechanism of regulation that cannot be over-looked is that MA and NS-398 could be decreasing the stimulation of 11 β HSD activities by increasing the metabolism of AA to epoxygenase and lipoxygenase products. The inhibition of PGHS by MA and NS-398 would increase the flux of AA through the epoxygenase and lipoxygenase pathways, the products of which could be inhibiting hCG- and IL-1 β - stimulated 11 β HSD activities.

In conclusion, this study has found that hCG and IL-1 β can increase 11 β HSD activities independent of changes in 11 β HSD1 protein expression. Co-treatment with NS-398 and MA abolishes the increase in the oxidative activities of 11 β HSD1 in response to both hCG and IL-1 β , both of which were confirmed to increase PGE₂ and PGF_{2 α} production. Taken together, these data suggest a possible role for eicosanoids in mediating hCG and IL-1 β stimulation of 11 β HSD activities in human granulosa-lutein cells.

**Chapter Seven: Elucidation of structural topology, regulatory domains, and
tertiary structure of 11 β HSD1**

Chapter 7: Elucidation of structural topology, regulatory domains, and tertiary structure of 11 β HSD1

7.1 Introduction

A number of studies have investigated the structure, localisation and topology of 11 β HSD1. In the early 1990's, Tsigelny and Baker [1995a; 1995b] generated the first structural domain prediction for 11 β HSD1 based on evolutionary links of 11 β HSD1 with other members of the short-chain alcohol dehydrogenase (SCAD) family of enzymes. In order to predict the structure of 11 β HSD1, bacterial 20 β HSD was selected as a template protein. By analogy, these authors concluded that (i) 11 β HSD1 dimerises, (ii) the active sites of 11 β HSD1 would be located within a bundle of 4 hydrophobic helices, and (iii) 11 β HSD1 contained an NADPH co-factor binding site within a Rossman fold [Tsigelny and Baker 1995a; 1995b].

Following on from these studies, Ozols generated the first topology predictions of 11 β HSD1 orientation within the smooth endoplasmic reticulum (ER) [Ozols 1995; Mziaut *et al*, 1999]. These predictions were based on several factors: the homology of the N terminus of 11 β HSD1 to the N terminus of the 50kDa esterase enzyme, the presence of 3 N-linked glycosylation sites that were presumed to be orientated towards the lumen of the ER, the presence of 2 cysteine residues for the formation of an intramolecular disulphide bond, (also assumed to adopt a luminal orientation), and that 11 β HSD1 was resistant to protease digestion unless the smooth ER membrane was disrupted using detergents. Based on homology to 50kDa esterase,

11 β HSD1 was predicted to have a very small cytosolic N terminus (Met₁-Lys₅), a single transmembrane domain (Lys₆-Glu₂₅), with the majority of the C terminus (Glu₂₆-Lys₂₉₂) inside the lumen of the ER with a hydrophobic core [Ozols 1995].

Subsequent biochemical studies further refined the models generated by Tsigelny and Baker, and by Ozols. Studies were conducted using FLAG epitopes at the extreme N and C termini of 11 β HSD1, in combination with detergents that selectively permeabilise only the plasma membrane, or both the plasma membrane and the ER membrane. These studies confirmed that the extreme N terminus of 11 β HSD1 does indeed reside in the cytosol and that the extreme C terminus is located in the lumen of the smooth ER. Investigations using site-directed mutagenesis revealed that Lys₅ was essential to maintain luminal orientation of the C terminus, as when this Lys was mutated to a Ser residue, the topology of 11 β HSD1 was reversed [Odermatt *et al.*, 1999].

Subsequent studies used site-directed mutagenesis to truncate the N terminus of 11 β HSD1. Mutants lacking either the N-terminal amino acids (aa) 1-15 or aa 1-30 revealed that these residues, encoded by exon 1 of the *HSD11B1* gene, are not essential to anchor 11 β HSD1 to the smooth ER membrane. However, when the truncated 11 β HSD1 proteins were also mutated at Val₁₄₉ to Arg, the truncated protein was partially released from the smooth ER membrane. On mutation of Val₁₄₉ to Glu, the truncated 11 β HSD1 was fully released from the ER membrane, localising exclusively to the cytosolic supernatant fraction. The hydropathy plot for 11 β HSD1, using the thermal modelling and analysis programme (TMAP), revealed that there

are 3 possible hydrophobic domains in human 11 β HSD1, the first being the predicted transmembrane domain (Met₄-Tyr₂₁), with a second hydrophobic domain just prior to the catalytic site (Val₁₃₆-Leu₁₅₈), and the third just after the catalytic site (Ile₁₉₇-Thr₂₂₀). Site-directed mutagenesis of Val₁₄₉ to Glu would reduce the hydrophobicity of the region prior to the active site, rendering it unable to penetrate the membrane as a transmembrane domain [Blum and Maser 2003].

Although the biochemical and *in silico* models of 11 β HSD1 topology appear to favour 11 β HSD1 as comprised of a single transmembrane domain with a short cytosolic N terminus and a large globular C terminal domain within the lumen of the ER, there are several problems with this model. The first is that when a mutant form of 11 β HSD1 was generated that lacked the 'first' transmembrane domain, the enzyme still co-precipitated with the smooth ER membrane. The second is that the co-factor binding site for NADPH is predicted to reside within the lumen of the ER, but the enzyme is known to respond to addition of exogenous pyridine nucleotides on the cytosolic side of microsomes. Therefore, the aims of the studies described in this chapter were to use multiple, complementary sequence analysis programs to develop alternative models for the 11 β HSD1 enzyme based on:

- i) Secondary structure prediction
- ii) Hydropathy plots (+/- topology predictions)
- iii) Predicted protein folds and domains

Since the primary sequence of bovine 11 β HSD3 has recently been deposited in the Entrez-Protein database, the sequence identities between 11 β HSD1, 11 β HSD2 and

11 β HSD3 have been compared in the cow (the only species in which all 3 11 β HSD enzymes have been cloned to date).

7. 2 Experimental Design

For all results obtained, the primary sequences for 11 β HSD1 were used. The complete primary amino acid sequences for 11 β HSD1 were obtained from NCBI Entrez Protein database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>).

7.2.1 Primary sequence alignment

Primary sequences for 11 β HSD1 were obtained for the following species: human (*Homo sapiens*), squirrel monkey (*Samiri sciureus*), pig (*Sus scrofa*), cow (*Bos taurus*), sheep (*Ovis aries*), rabbit (*Oryctolagus cuniculus*), guinea pig (*Cavia porcellus*), hamster (*Mesocricetus auratus*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*), and the partial sequence to date for cat (*Felis catus*). All sequences were converted to FASTA format, and sequence alignments were performed. Two different methods for alignment were used, the first being based on manual sequence alignments to the human 11 β HSD1 sequence by visual inspection and the second using the CLUSTALW v1.4 database (obtained from <http://pir.georgetown.edu/pirwww/search/multaln.html>). CLUSTALW is a progressive alignment method that builds up a multiple sequence alignment starting with the most related sequences, and then by adding the less related sequences to the original alignment. The order in which the sequences are added is based on an initial

all by all pairwise alignment, therefore generating an un-biased alignment of the sequences.

This methodology was also used to determine the sequence homology between the bovine 11 β HSD1, 11 β HSD2 and 11 β HSD3 proteins.

7.2.2 Functional site characterisation of the 11 β HSD1 primary sequence

Potential candidates for functional sites within the 11 β HSD1 sequence were identified on the basis of the presence of consensus sequences for such domains using the Prosite database (<http://ca.expasy.org/prosite>).

7.2.3 Secondary structure predictions

Secondary structure predictions were made using PsiPred version 2.0 (<http://bioinf.cs.ucl.ac.uk/psipred/>) [Jones 1999; McGuffin *et al.*, 2000].

7.2.4 Localisation of TM domains

Once sequence alignments were complete, hydrophobicity plots were generated for the aligned proteins initially using Kyte-Doolittle scores. When deriving the hydropathy plots the window size was set to 9 amino acids, this being the optimal size when looking for surface regions in a globular protein. The window size is the number of amino acids whose hydrophobicity scores are averaged and assigned to the middle amino acid in the window. Since the hydrophobicity plots for all 11 species were very similar, all subsequent studies were performed using only the primary and secondary structure predictions for human 11 β HSD1.

The human sequence for 11 β HSD1 was analysed using five transmembrane prediction tools. Five different predictive tools were used: TMAP [Persson and Argos, 1997], Topology Predictor (TopPred) [Claros and von Heijne, 1994] using Kyte Doolittle (KD) scores, TopPred using Goldman Engelman Steitz (GES) scores, model recognition (MEMSAT 2) [Jones 1998; Jones 1994] and transmembrane helical model (TMHMM) [Krogh *et al.*, 2001]. Transmembrane domains were predicted based on the hydrophobicity of each amino acid residue within the sequence and the hydrophobicity of surrounding residues.

7.2.5. Tertiary structure prediction- Fold recognition

Using the output from the database combined with topology predictions, 3 alternative models for the tertiary structure of human 11 β HSD1 were generated. Modified genomic threading (mGenTHREADER) (<http://bioinf.cs.ucl.ac.uk/psipred/>) [McGuffin and Jones 2003; Jones 1999] was used to predict functional folds within the protein based on homology to other protein folds within the mGenTHREADER database. The best templates for fold homology to 11 β HSD1 were predicted based on homology to 11 β HSD1 without the first predicted transmembrane domain. Criteria that were used to assess the templates were low energy scores (solvation energy, pairwise energy, and the net energy) which provide a co-efficient (E scores) to predict the probability of fold homology to 11 β HSD1.

7.3 Results

7.3.1. Alignment of 11 β HSD1 primary sequences

Primary sequence alignments for all published 11 β HSD1 sequences were conducted by visual inspection (Figure 7.1A) and using CLUSTALW (Figure 7.1B) and subsequently aligned in terms of identity to the human sequence of 11 β HSD1. Analysis of percentage sequence homology across species revealed that the 11 β HSD1 has remained highly homologous. The N terminal sequence upstream of the active site exhibits greater than 80% sequence identity across the mammalian species. The C terminal region downstream of the active site was less homologous, with a minimum matching sequence identity of 66.4% when comparing the mouse or rabbit sequences to the human 11 β HSD1 sequence (Table 7.1).

7.3.2. Alignment of bovine 11 β HSD1, 11 β HSD2 and 11 β HSD3 primary sequences

Primary sequence alignments for bovine 11 β HSD1, 11 β HSD2 and 11 β HSD3 sequences were carried out using CLUSTALW (Figure 7.2). Bovine 11 β HSD3 was found to share 44% residue identity when compared to bovine 11 β HSD1. 11 β HSD2 shared less identity, with 26% of residues identical to bovine 11 β HSD1.

7.3.2 Analysis of functional sites and regulatory regions in all sequences aligned of 11 β HSD1

In all 11 species, the consensus sequence for SCAD enzymes (YxxxK) was conserved (Figure 7.3). In human 11 β HSD1, this consensus sequence lies at amino

acid positions 183-187 (YSASK), with the catalytic region spanning Val/Ala₁₆₆ to Phe₁₉₄. The NADP(H) co-factor binding site (spanning residues Val₄₇ to Leu₈₁ in 11 β HSD1) was also highly conserved (>83% identity) between species. Three conserved sites for N-linked glycosylation were predicted across species (Figure 7.3). In human 11 β HSD1, two cysteine residues (Cys₇₈-Cys₂₁₃) were conserved, and are thought to take part in intramolecular disulphide bond formation. Possible sites for myristoylation of the enzyme were also identified. Several consensus sequences were located for regulation by kinases including PKC, Casein Kinase-2 (CK2) and tyrosine kinase. These possible phosphorylation sites were generally located downstream (C terminally) with respect to Glu₂₅.

7.3.3. Secondary Structure prediction for human 11 β HSD1

The secondary structure of human 11 β HSD1 was predicted by PsiPred to consist of 11 α helices and 6 β strands interlinked by random coils (Figure 7.4).

7.3.4. Topology predictions for 11 β HSD1

A hydropathy plot of the aligned 11 β HSD1 sequences, using the KD scores, revealed that across all species the topology of the protein is highly conserved (Figure 7.5). All species contain 5 regions of hydrophobicity, with 3 of sufficient hydrophobicity and sequence length to span the membrane: The first of the three putative transmembrane domains is located at the N terminus of the protein, and regions 2 and 3 flank the enzyme's active site.

To assess the reliability of the topology as predicted by a hydropathy plot conducted using KD scores, further predictions were carried out using a total of 5 different programs. Each program used presented different models for the topology of 11 β HSD1 (Figure 7.6). TMAP predicted that 11 β HSD1 contains 3 transmembrane domains at Met₄ to Tyr₂₁, Val₁₃₆-Leu₁₅₈ and Ile₁₉₇-Thr₂₂₀. If Goldman Engelman Steitz (GES) hydrophobic scores were used, TopPred also predicted 11 β HSD1 to contain 3 transmembrane domains although at positions Met₄ – Asn₂₄, Val₁₃₆ – Leu₁₅₈, and Glu₁₆₃ – Tyr₁₈₃. Further analysis using TopPred with the KD scale of hydrophobicity predicted 5 transmembrane domains at Met₁-Tyr₂₁, Phe₁₀₂-Thr₁₂₂, Ser₁₃₉-Lys₁₅₉, Asn₁₆₂-Ala₁₈₂, and Tyr₂₀₁-Thr₂₂₀. Both MEMSAT2 and TMHMM programs predicted only a single membrane spanning region at Tyr₇-Tyr₂₁ or Tyr₇-Ser₂₂, respectively.

To elucidate the role of Val₁₄₉ in stabilising the hydrophobic domains as shown by Odermatt *et al* [2001], TMAP-KD was used, but with Val₁₄₉ replaced with either Arg or Glu. Replacing the hydrophobic Val at position 149 with Arg or Glu decreased the hydrophobicity scores of the surrounding regions. A decrease in overall hydrophobicity of the protein was observed with this mutation, specifically the affected region spanned from Leu₁₄₅ to Ala₁₅₃.

7.3.5. Tertiary structure models

Combining the results of the secondary structure and topology predictions, 3 possible models for the tertiary structure of human 11 β HSD1 were generated. The models generated make the assumption that the in vitro studies carried out by

Odermatt *et al*, [2001] to determine the orientation of the extreme N and C terminus of 11 β HSD1 in the smooth ER membrane were correct. Therefore all models are generated on the assumption that the extreme N terminus of 11 β HSD1 is localised to the cytoplasm, and the extreme C terminus is localised to the lumen of the ER. Model 1 predicts a single transmembrane protein, that is able to form a dimerisation interface through α helix_E, with a potential inter-molecular disulphide bond at Cys₂₇₂ (Figure 7.7a). The N terminus of the protein is localised to the cytosol, with α -helix_A forming a membrane spanning region, and the C terminus within the lumen of the ER. Both the catalytic site (β strand_E and α helix_F) and the Rossman fold that constitutes the NADP(H) co-factor binding site (β strand_A to α helix_C) are localised to the lumen of the ER.

Model 2 predicts a protein with 3 transmembrane domains that forms a dimer (Figure 7.7b). Again, the N terminus of 11 β HSD1 is localised to the cytosol with α helix_A forming the first putative membrane spanning region. The second transmembrane region is through α helix_E, and the third is through α helix_F. In model 2, the active site of 11 β HSD1 and the cofactor binding site are both located within the hydrophobic core of the smooth ER membrane.

Model 3 also predicts that 11 β HSD1 has 3 transmembrane domains (Figure 7.7c). As in models 1 and 2, the N terminus is localised to the cytosol with the C terminus within the lumen of the ER. α helix_A comprises the first putative transmembrane domain with α helix_E and α helix_F plus β strand_F forming the second and third transmembrane domains, respectively. The active site is localised to the cytosol

while the NADP(H) co-factor binding domain is localised to the lumen of the ER. The protein is speculated to form homodimers at the third transmembrane domain with a potential intermolecular disulphide bond at Cys₂₇₂ to stabilise the dimer.

To assess the validity of each of these 3 models, a number of questions were generated, based on features that were deemed essential for 11 β HSD1 to be functional (Table 7.2). The questions asked of each model were:

- (i) Are Cys₇₈, Cys₂₁₃ and Cys₂₇₂ localised to the lumen of the ER for intra- and inter-molecular disulphide bond formation respectively?
- (ii) Are Asn₁₂₃, Asn₁₆₂ and Asn₂₀₇ located in the lumen of the ER for N-linked glycosylation?
- (iii) Are the NADP(H) co-factor binding site and catalytic active site located on the same side of the ER membrane?
- (iv) Are Ser₁₆₉ and Tyr₁₈₃ within close proximity to form the catalytic site of 11 β HSD1?

The intra-molecular disulphide bond proposed between Cys₇₈ and Cys₂₁₃ [Ozols 1995] would be promoted in the oxidative environment of the ER lumen, as would the inter-molecular bond (at Cys₂₇₂) that is hypothesised to stabilise the dimer interface specifically for human 11 β HSD1. In models 1 and 2, all 3 Cys residues are located within the lumen of the ER, but in model 3, Cys₂₁₃ is localised within the ER membrane. This would render the possibility of the formation of an intramolecular disulphide bond in model 3 unlikely.

11 β HSD1 is also known to contain 3 sites for N-linked glycosylation, which can only occur within the lumen of the ER. Therefore each of the Asn residues at position 123, 162 and 207 should be located within the lumen of the ER. Model 1 is the only model that has all 3 Asn residues located within the lumen of the ER. Model 2 has Asn₁₆₂ within the cytosol, and model 3 predicts that Asn₁₆₂ and Asn₂₀₇ would be localised to the cytosol and hydrophobic core of the smooth ER membrane respectively.

For 11 β HSD1 to be functional, both the catalytic site and co-factor binding site would need to be on the same side of the membrane. Model 2 would orientate the catalytic site and the cofactor binding site on opposite sides of the membrane. Model 3 would localise the active site within the ER membrane and the co-factor binding site to the lumen of the ER. In contrast, Model 1 localises both the catalytic and cofactor binding site to the lumen of ER.

The key catalytic residues of Ser₁₆₉ and Tyr₁₈₃ of the catalytic triad would need to be within close proximity for the active site to be functional [Tsigelny and Baker 1995b]. Ser₁₆₉ is necessary to decrease the pKa of Tyr₁₈₃ for catalysis. In Model 2 the Tyr and Ser residues would not be able to contact each other to form the key catalytic centre for cortisol-cortisone inter-conversion, such that Model 2 would almost certainly not produce a functional enzyme.

Taking all of these data into consideration, Model 1, appears to fulfil all criteria necessary for the 11 β HSD1 enzyme to be functionally viable. Therefore 11 β HSD1 appears to be a single transmembrane protein orientated with the extreme N terminus

Figure 7.1A: Visual alignment of primary sequences of 11 β HSD1 from other mammalian species to human 11 β HSD1

	1	10	20	30	40	50
Homo sapiens	MAFMKKYLLPILGLFMAYYYYSANEEFRPEMLQGKKVIVTGASKGIGREM					
Saimiri sciureusTH.....Y.....					
Sus scrofaI..L.....R.....					
Bos taurusI..L.....R..R.....					
Ovis ariesL.....R..R.....					
Felis catusEQ.					
Oryclolagus cuniculus	*.....L....L.....K.I					
Cavia porcellus	...L....T..MV..L....P...K.....I					
Mesocricetus auratus	...L.....V..L....TK.....					
Rattus norvegicusCLG*...T.....					
Mus musculus	..V..N.....V.SL....T.....					

	60	70	80	90	100
Homo sapiens	AYHLAKMGAAHVVTARSKETLQKVVSHCLELGAASAHYIAGTMEDMTFAE				
Saimiri sciureus				
Sus scrofaR.....E...K....S....S...V.....				
Bos taurusR.....E.S.K....R.....V....N....				
Ovis ariesR.....E.S.K....R.....V....N....				
Felis catusE..A...K.....				
Oryclolagus cuniculus	.F.....A....AR.....S.....				
Cavia porcellusA....AR.....S.....				
Mesocricetus auratus	...SE.....L...E.G....A.R.....				
Rattus norvegicus	...S.....L...E.G....R.....				
Mus musculus	...S.....L...E.G....R.....				

	110	120	130	140	150
Homo sapiens	QFVAQAGKLMGGDLMLILNHITNTSLNLFHDDIHHVRKSMEVNFLSYVVL				
Saimiri sciureusF.....				
Sus scrofa	...K...L.....QAFMTSVY...L..R.....				
Bos taurus	...K..E.V.....HY.P.GV.SN...LL.RTL...L.....				
Ovis aries	...K..E.V.....NY.P.RV.SN...LL.R.L...L.....				
Felis catus	...K.....M...SG...I..R.....				
Oryclolagus cuniculus	...K.....A..MF.NN.....E.....				
Cavia porcellus	E.A.E..N.....VLYNR.TF..GE.DN.....H.F...				
Mesocricetus auratus	...LK.....Y..M.F.R.E...L..A....I...M				
Rattus norvegicus	R..VE...L.....Q.TMS.....S..R.....				
Mus musculus	..IVK.....Q...S.....S..RV.....M				

	160	170	180	190	200
Homo sapiens	TVAALPMLKQSNGSIVVVSSLAGKVAYPMVAAYSASKFALDGFSSIRKE				
Saimiri sciureus	...M.....I...V.....IS.....Y.....				
Sus scrofa	S.....Q...M.N.L..P.....T..				
Bos taurus	ST.....T.....I...I.C.L.....L.M.				
Ovis aries	ST.....TS.....V...I.C.LA.....L.T.				
Felis catus	SAT.....L...M.S.LI.P.....M.				
Oryclolagus cuniculusI.H.LI.P.....A...				
Cavia porcellus	S...M...M.Q...A...V...IT..LI.P.....TL.S.				
Mesocricetus auratus	S.....I...M.H.L..S.....L.R.				
Rattus norvegicus	ST.....AII..M...MTQ.LI.S.....T...				
Mus musculus	ST.....A.I...MTQ..I.P.....T..T.				

	210	220	230	240	250
Homo sapiens	YSVSRVNV	SITLCVLGLIDTETAMKAVSGIVHMQAAPKEECALEI	IKGGA		
Saimiri sciureus	.LM.E.....	D.....	IK....	R.....	V
Sus scrofa	...TK.....	I.....	D.....	A..LNV.PS.....	A.
Bos taurus	.EATK.....	I.....	D.....	A..FNAK.S.....	T
Ovis aries	.EATK.....	I.....	D.....	A..YNAE.S....	**.....
Felis catus	Y..SRV.....	I....	N.D...N.I..		
Oryzomys rickettsii	HALTN.....	D....	E...KID.K.S.....		
Cavia porcellus	FL.NK.....	I.....	I..T...YLGP.S.....		T.
Mesocricetus auratus	HG.TN.....	N.....	T..VFNAK.S.....		
Rattus norvegicus	HLMTK.....	F.....	L.ET...ILS....	Q.....	*..TV
Mus musculus	LYITK.....		EI...IDAL.S.....		T.

	260	270	280	290	300
Homo sapiens	LRQEEVYYDSSLWTTLLIRNPCRKILEFLYSTSYNMDRFINK	-----			
Saimiri sciureusR.....	L...S.....	R....	ST.GL..	-----
Sus scrofaIL.S..	LE..G.....	SLRH...	E..T.N	-----
Bos taurus	...D.....	IL.P..L...G...M..	FFLKK...	E....N	-----
Ovis aries	...D.....	N.IL.S..LK..G...M..	SLKK...	E....N	-----
Oryzomys rickettsii	...D....	GNLQ..P..LG..G..LI...	HLRKF	DISKLV.N	-----
Cavia porcellus	...D.M..	VG.R.VPY.LG..G...M...	SAAE..W.NVLSN	EKLYGRWA	
Mesocricetus auratusWS..	PI.LG..G...M...	SMK.FTF.KL.SS	-----	
Rattus norvegicus	..KD.....	K.P..P..LG..G.R.M...	SLR...R.L.VSN	-----	
Mus musculus	..KS.....	KLPL.PI.LG..G...M..	FSLRY..K.M.VSN	-----	

Figure 7.1B: Primary sequence alignment of 11 β HSD1 from different mammalian species using CLUSTALW database and subsequent alignment to the human enzyme in order of evolutionary divergence

	1	10	20	30	40	50
Homo sapiens	MAFMKKYLLPILGLFMAYYYYSANEEFRPEMLQGGKVI	VTGASKGIGREM				
Saimiri sciureusTH.....Y.....				
Sus scrofaI.L.....R.....				
Bos taurusI.L.....R..R.....				
Ovis ariesI.L.....R..R.....				
Felis catus	-----	-----EQ.			
Oryzolagus cuniculus	-.....L.....K.I				
Cavia porcellus	..L.....T..MV.L.....I				
Mesocricetus auratus	.H.....V..L.....TK.....					
Rattus norvegicus	---.....V.V.CLG-.....T.....					
Mus musculus	..V..N.....V.SL.....T.....					

	60	70	80	90	100
Homo sapiens	AYHLAKMGAHVVTARSKETLQKVVSHCLELGAASAHYIAGTMEDMTFAE				
Saimiri sciureus				
Sus scrofaR.....E...K....S....S...V.....				
Bos taurusR.....E.S.K....R.....V....N.....				
Ovis ariesR.....E.S.K....R.....V....N.....				
Felis catusN.K.I...M.....N.....				
Oryzolagus cuniculusE..A...K.....				
Cavia porcellusA....AR.....S.....				
Mesocricetus auratus	...SE.....L...E.G....A.R.....				
Rattus norvegicus	...S.....L...E.G....R.....A...				
Mus musculus	...S.....L...E.G....R.....				

	110	120	130	140	150
Homo sapiens	QFVAQAGKLMGGDLMLILNHTNTSLNLFHDDIHHVRKSMEVNFLSYVVL				
Saimiri sciureus				
Sus scrofa	...K...L.....QAFMTSVY...LV.R.M.....				
Bos taurus	...K..E.V.....HY.P.GV.SN...LL.RTL...L.....				
Ovis aries	...K..E.V.....NY.P.RV.SN...LL.R.L...L.....				
Felis catus	...K.....M...SG...I..R.....				
Oryzolagus cuniculus	...K.....A...F.NN.....E.....				
Cavia porcellus	E...E..N.....VLYNR.TF..GE..NV.K.M...H.F...				
Mesocricetus auratus	...LK.....Y..M.F.R.E..AL..A...I...M				
Rattus norvegicus	R..VE...L.....Q.TMS.....S..R.....				
Mus musculus	..IVK.....Q...S.....S..RV.....M				

	160	170	180	190	200
Homo sapiens	TVAALPMLKQSNISIVVSSLAGKVAYPMVAAYSASKFALDGGFFSSIRKE				
Saimiri sciureus	...M.....I..V.....IS.....Y.....				
Sus scrofa	S.....Q...M.N.L..P.....ITK.				
Bos taurus	ST.....T.....I...I.C.L.....L.M.				
Ovis aries	ST.....TS.....V...I.C.LA.....L.T.				
Felis catus	SAT.....K...M.S.LI.P.....M.				
Oryzolagus cuniculusI.H.LI.P.....A...				
Cavia porcellus	S...M...M.Q...A...V...IT..LI.....TL.S.				
Mesocricetus auratus	S.....I...M.H.L..S.....L.R.				
Rattus norvegicus	ST.....AII..M...MTQ.LI.S.....T...				
Mus musculus	ST.....A.I.....MTQ..I.P.....T..T.				

	210	220	230	240	250
Homo sapiens	YSVSRVNV	SITLCVLGL	IDTETAMK	AVSGIVHMQ	AAPKEECALEI
Saimiri sciureus	.LM.E.....	D.....	IK.....	V.....	
Sus scrofa	.S.TK.....	I.....	D...T..A..	LNVP.S.....	
Bos taurus	.EATK.....	I.....	D.....	A..FNAK.S.....	T
Ovis aries	.EATK.....	I.....	D.....	A..YNAE.S.....	
Felis catus	H..AKI.....	I....N.D...	N.I.....		
Oryzomys rickettsi	HALTN.....	D....E...	KID.K.....		
Cavia porcellus	FL.NK.....	I.....	I..T...YLGP.S.....		T.
Mesocricetus auratus	HG.TN.....	N.....	T..VFNAK.S.....		
Rattus norvegicus	HLMTK.....	F.....	L.ET...ILS.....	Q.....	-...TV
Mus musculus	LYITK.....		EI...IDAL.S.....		T.

	260	270	280	290	300
Homo sapiens	LRQEEVYYD	SSLWTTLL	IRNPCRKILE	FLYSTSYNMD	RFINK-----
Saimiri sciureusR.....	L...S.....	R....ST.GL..		-----
Sus scrofaIL.S..	LE..G.....	SLRH..ME..T.N		-----
Bos taurus	...D.....	IL.P..L...G...	M..FFLKK...E...	N-----	
Ovis aries	...D.....	N.IL.S..LK..G...	M...SLKK...E...	N-----	
Oryzomys rickettsi	...D....G	NLQ..P..LG..GKRLI...	HLRKFDISKLV.N		
Cavia porcellus	...D.M...G	R.VPY.LG..G...M...	SAAE..W.NVLSNEKLYGRWA		
Mesocricetus auratusWS..	PI.LG..G...M...	SMK.FTF.KL.SS-----		
Rattus norvegicus	..KD.....	K.S..P..LG..G.R.M...	SLR...R.L.VSN-----		
Mus musculus	..KS.....	KLPL.PI.LG..G...M...	FSLRY..K.M.VSN-----		


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1         10         20         30         40         50
-----MAFMKKY
11 beta HSD 1 Bos taurus MESWPWPSPGGAWLLVPARALLQLLRADLRRLGRPLLAALALLAALDWLCQR
11 beta HSD 2 Bos taurus -----
11 beta HSD 3 Bos taurus -----..VL

        60         70         80         90        100
LLP---ILGIFLAYYYYSANEEFRPEMLRGKR--VIVTGASKGIGREMA
11 beta HSD 1 Bos taurus ...PLAA.AVLA.TGWIVLSRLA..QR.PVATRA..I..CDSGF.NAT.K
11 beta HSD 2 Bos taurus ..T--G..ALFFA..WDD.--.D.AS.H.A.--.LL..V.A...E.L..
11 beta HSD 3 Bos taurus

        110        120        130        140        150
HLARMGAHVVTARSEESLKKVVSRCLELGAASAHYVAGTMENMTFAEQF
11 beta HSD 1 Bos taurus K.DT..FT.LA.VLDLN.PGALEL.ACCSSRLKLLQMDL.KPGDISRVLE
11 beta HSD 2 Bos taurus .Y..L.S.L.L..HT.AL.QQ..GN.RK...PKVF.I.AD.ASPEVP.RV
11 beta HSD 3 Bos taurus

        160        170        180        190        200
VAKAGELVGGDLMLILNH-IHYTPLGVFSNDIHLRRTLEVNLISYVVLS
11 beta HSD 1 Bos taurus FT.VHTPST..WG.VN.AGQNI FVADAELCPVATF.TCM...FFGALEMT
11 beta HSD 2 Bos taurus .QF.LDKL....Y.V...-LGAA.A.TRVRSQST.WLMQM.F....Q.T
11 beta HSD 3 Bos taurus

        210        220        230        240        250
TAALPMLKQTNGSIVVSSIAGKACPLVAAYSASKFALDGGFFSSLRMEY
11 beta HSD 1 Bos taurus KGL..L.RRSS.R..T...P..DMPF.CL...GT..A..ALLMGNFSC.L
11 beta HSD 2 Bos taurus SS...S.TDSK..L....LL.RVPTSFSSP...A.....S.....R.L
11 beta HSD 3 Bos taurus

        260        270        280        290        300
EATKVNVSIT----LCILGLIDTDTAMKAVAGIFN-----
11 beta HSD 1 Bos taurus LPWG.K...IQPACFKTESVK.VHQWEERKQQLLATLPQELLQAYGEDYI
11 beta HSD 2 Bos taurus DVQE...A..---M.V...R.RAS.AEG.R..TR-----
11 beta HSD 3 Bos taurus

        310        320        330        340        350
-----AKASPKEECALEIIKGGTLRQD--EVYYDSS-ILTPLLL
11 beta HSD 1 Bos taurus EHLNGQFLHLSLSQ.L.DLSPVDA.TDAL.AAQPLRR..PGHGLGLIYFI
11 beta HSD 2 Bos taurus -----VR.A.GPKA..AV.R..ATRAS--G.F.PWR-FHLLC..
11 beta HSD 3 Bos taurus

        310        320        330        340        350
RNPGRKIMEFFFLKKYNMERFINN-----
11 beta HSD 1 Bos taurus HYYLPEGLRQR..QSFFISPYVPRALQAGQPGLTSARDIAQDQGPRPDPS
11 beta HSD 2 Bos taurus .SWMPHSRAW.VRQELNITTPAAA-----
11 beta HSD 3 Bos taurus

11 beta HSD 1 Bos taurus ----
11 beta HSD 2 Bos taurus PTAQ
11 beta HSD 3 Bos taurus ----

```

Figure 7.2: Primary sequence alignment of bovine 11 β HSD1, 11 β HSD2 and 11 β HSD3 using CLUSTALW database.

Figure 7.3: Predicted conserved regulatory sites of 11 β HSD1. PTM= post-translational modification, N-myristoylation, CK2= casein kinase 2 phosphorylation site, diS= disulphide bond linkage, N-glyco=N-linked glycosylation sites, Amid=amidation.

PTM			N-Myr		CK2		Amid.	
Kinase								
Human	1	MAFMKKYLLPIL	GLFMAYYYY	SANE	EFRPEML	QGKK		VIVTGASKGIGREM
Monkey	1TH.....Y.....
Pig	1	I.L.....	R.....	
Cow	1	I.L.....	R..R	
Sheep	1	L.....	R..R	
Cat							EQ.....
Rabbit	1	*L.....L.....K.I.....
Guinea	1	..L.....T..MV.L.....	P..K.....I.....
Hamster	1	.H.....V..L.....	TK.....I.....
Rat	1V.V.CLG*	T.....
Mouse	1	..V..N.....V..L.....	T.....
Site / Domain		Transmembrane domain						NADP(H) Binding Site

PTM			PKC	PKC	diS	N-Myr	N-Myr	N-Glyco.	CK2	CK2
Kinase										
Human	51	AYHLAKMGAHVVV	TARSKETL	QKVVSH	CLEL	GAASAHYIAG	TMED	MTFAE		
Monkey	51
Pig	51R.....E..	K.....S.....	S.S..V.....
Cow	51R.....E..S.	K.....R.....V.....N.....
Sheep	51R.....E..S.	K.....R.....V.....N.....
Cat	N.	K.I.....M.....N.....
Rabbit	50	.F.....E..A.....K.....
Guinea	51A.AR.....	S.....
Hamster	51SE.....L.....E.G.A.R.....
Rat	47S.....L.....E.G.R.....
Mouse	51S.....L.....E.G.R.....
Site / Domain		NADP(H) Binding Site								

PTM

N-Glyco.

PKC

Kinase

Human	101	QFVAQAGKLMGGLDMLILNHIT	NTSL	NLFHDDIHHVRKSMEVNFLSYVVL
Monkey	101F
Pig	101K....L.....	QAFMTSVYL..R.....
Cow	101K..E.V.....	HY.P.GV.SN...	LL.RTL...L.....
Sheep	101K..E.V.....	NY.P.RV.SN...	LL.R.L...L.....
Cat	101K.....M...SG...I..R.....
Rabbit	100K.....A...MF.NN.....	E.....
Guinea	101	E.A.E..N.....	VLYNR.TF..GE.DN.....	H.F...
Hamster	101	...LK.....	Y..M.F.R.E..AL..A.....	I...M
Rat	97	R..VE....L.....	Q.TMS.....	<u>S</u> ..R.....
Mouse	101	..IVK.....	Q...S.....	<u>S</u> ..RV.....M

PTM

N-Glyco

PKC

Kinase

Human	151	TVAALPMLKQSN	NGSI	VVVSSLAGKVAYPMVAAYSASKFALDGFF	S	<u>SIRKE</u>
Monkey	151M.....I...V.....IS.....Y...
Pig	151	S.....Q...M.N.L..P.....T..
Cow	151	ST.....T.....I...I.C.L.....L.M.
Sheep	151	ST.....TS...V...I.C.LA.....L.T.
Cat	151	SAT.....K...M.S.LI.P.....M.
Rabbit	150I.H.LI.P.....	A....
Guinea	151	S...M...M..Q...	A...V...IT..LI.P.....TL.S.
Hamster	151	S.....I...M.H.L..S.....L.R.
Rat	147	ST.....	AII..M...MTQ.LI.S.....T....
Mouse	151	ST.....	A.I.....MTQ..I.P.....T...T.

Site /
Domain

Catalytic Site

PTM

N-Glyco. diS

N-Myr

N-Myr

Kinase

CK2

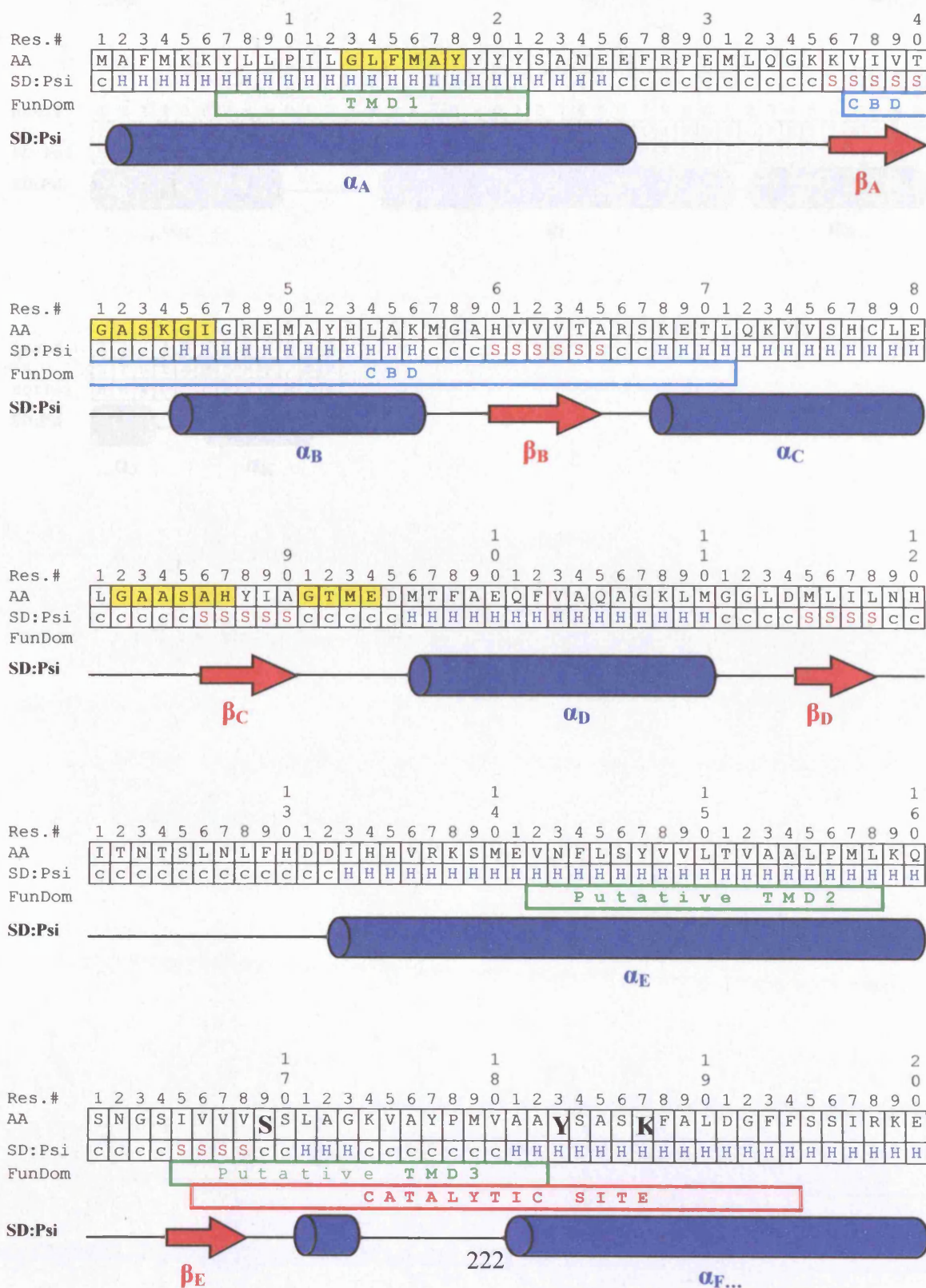
PKC+CK2

Human	201	YSVSRV	NVSI	TL	CVL	GLIDTE	TAMKAVSGIVHMQAAPKEECALEIIKGA
Monkey	201	.LM.E.DIK....R.....V
Pig	201	...TK.ID	...T..A..LNV.PS...A.
Cow	201	.EATK.IDA..FNAK. <u>S</u>T
Sheep	201	.EATK.IDA.. <u>YNAE</u> . <u>S</u>L**.....
Cat	201	H..AKIIN.D	...N.I..
Rabbit	200	HALTN.DE...KID.K.....
Guinea	201	FL.NK.II..T...YLGP. <u>S</u>T.
Hamster	201	HG.TN.I..T...FNAP. <u>S</u>
Rat	197	HLMTK.F	...L.ET.. <u>ILS</u>Q....*...TV
Mouse	201	LYITK.EI.. <u>INA</u> . <u>S</u>T.

PTM**diSAmid****Kinase****TyrK****PKC TyrK PKC**

Human	251	L	<u>RQEEVYY</u>	DSSLWTTLLIRNP	<u>CRKILEFLY</u>	STSYNMDRFINK	292
Monkey	251	R.....L...	<u>S</u>R.....	ST.GL..	291
Pig	251	IL.S..LE..	<u>G</u>	<u>SLR</u> H...E..T.N	292
Cow	251	.	<u>D</u>	IL.P..L...	<u>G</u> ...M..	FFLKK...E....N	292
Sheep	249	.	<u>D</u>	N.IL.S..LK..	<u>G</u> ...M...	<u>SLK</u> K...E....N	290
Rabbit	250	.	<u>D</u>	GNLQ..P..LG..	<u>GKRLI</u> ...	HLRKFDISKLV.N	291
Guinea	251	.	<u>D.M</u> ..	VG.R.VPY.LG..	<u>G</u> ...M...	SAAE...W.NVLSNEKLYGRWA	300
Hamster	251	WS..PI.LG..	<u>G</u> ...M...	SMK.FTF.KL.SS	292
Rat	246	..	KD.....	K.P..P..LG..	<u>G.R</u> ..M...	<u>SLR</u> ...R.L.VSN	287
Mouse	251	..	KS.....	K.PL.PI.LG..	<u>G</u> ...M..	F <u>SLRY</u> ..K.M.VSN	292

Figure 7.4: Secondary structure prediction for human 11 β HSD1 generated by PsiPred database based on PsiBLAST scores when compared to other proteins within the PsiPred database



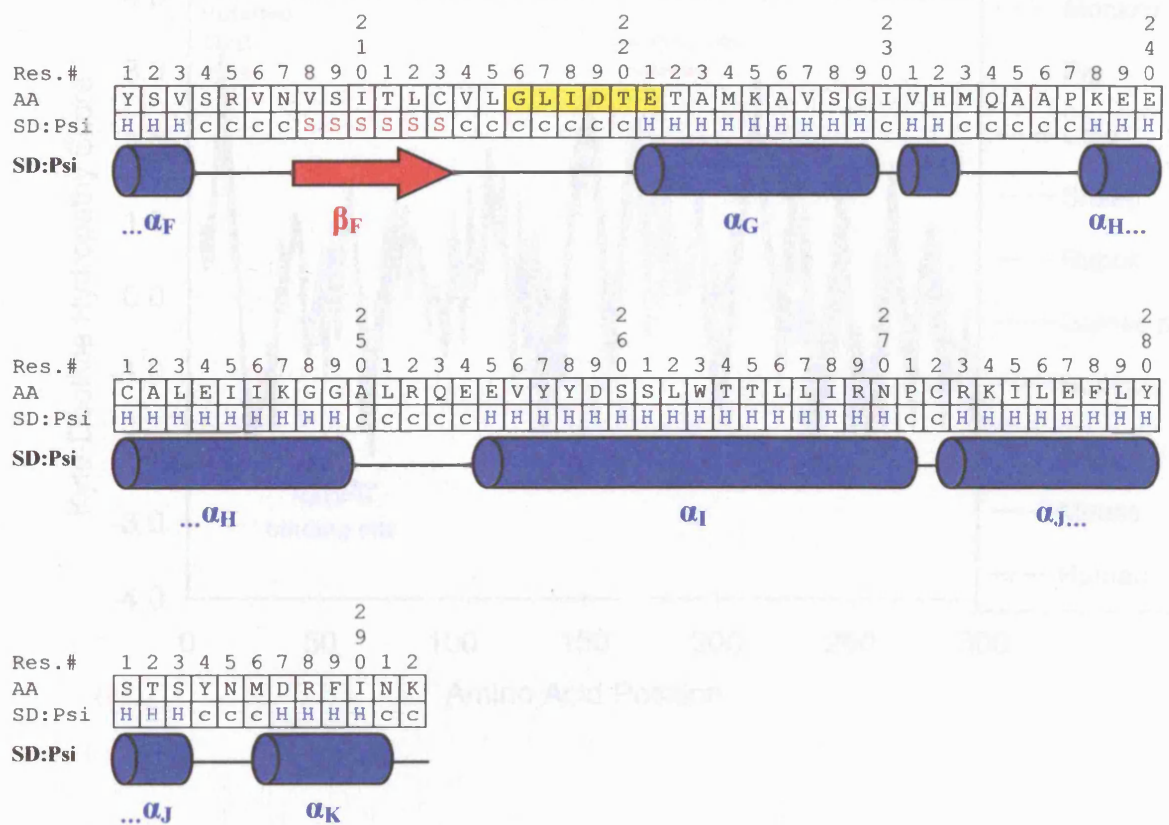


Figure 7.5: Hydropathy plot of aligned primary sequences for maximum two types of enzymes. The Kyte-Doolittle scale was used as a measure of hydrophobicity, with positive values being more hydrophobic and negative values being less hydrophobic.

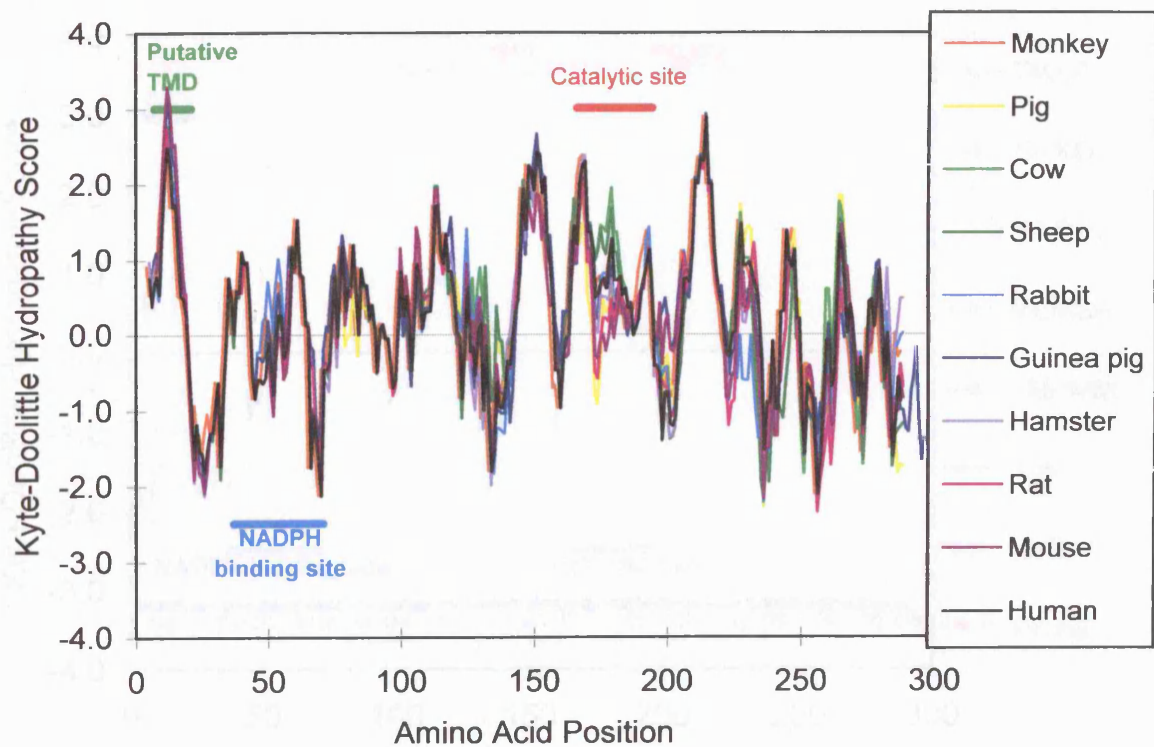


Figure 7.5: Hydropathy plot of aligned primary sequences for mammalian 11 β HSD1 enzymes. The Kyte-Doolittle scale was used as a measure of hydrophobicity with positive values being more hydrophobic and negative values being less hydrophobic.

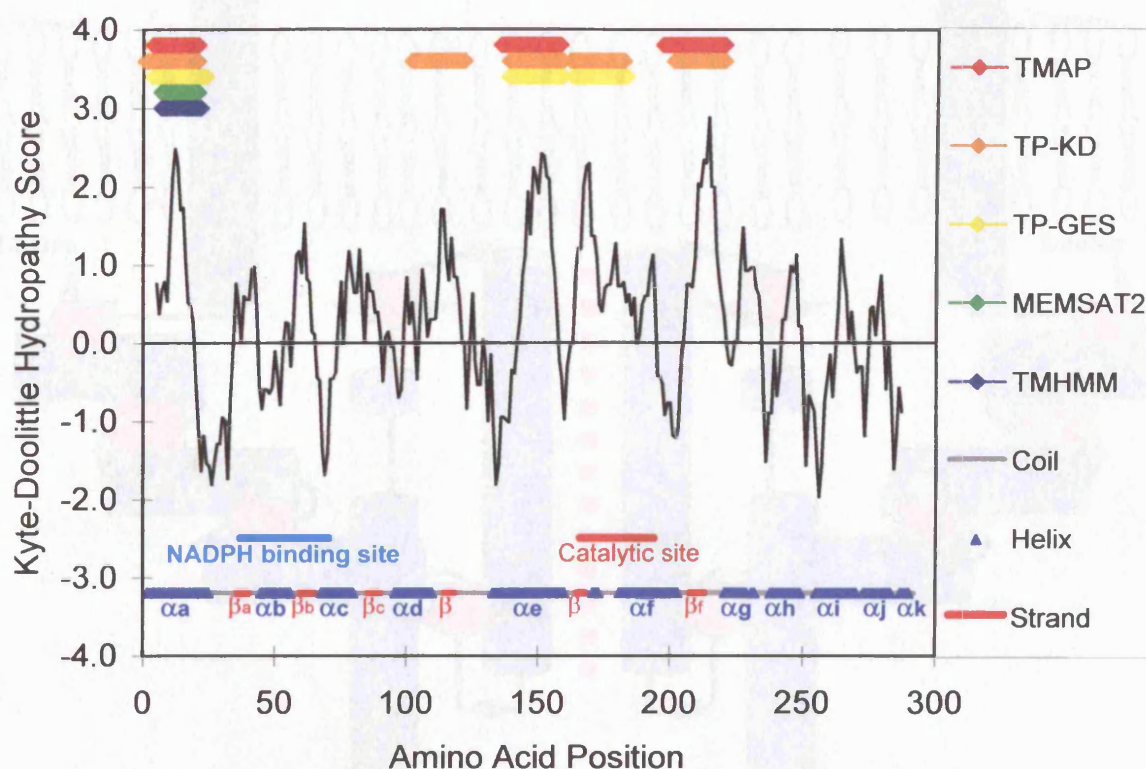


Figure 7.6: Hydropathy plot and transmembrane domain predictions for human 11βHSD1 using TMAP, TopPred-KD (Kyte-Doolittle scores), TopPred-GES, MEMSAT 2 and TMHMM topology prediction methods.

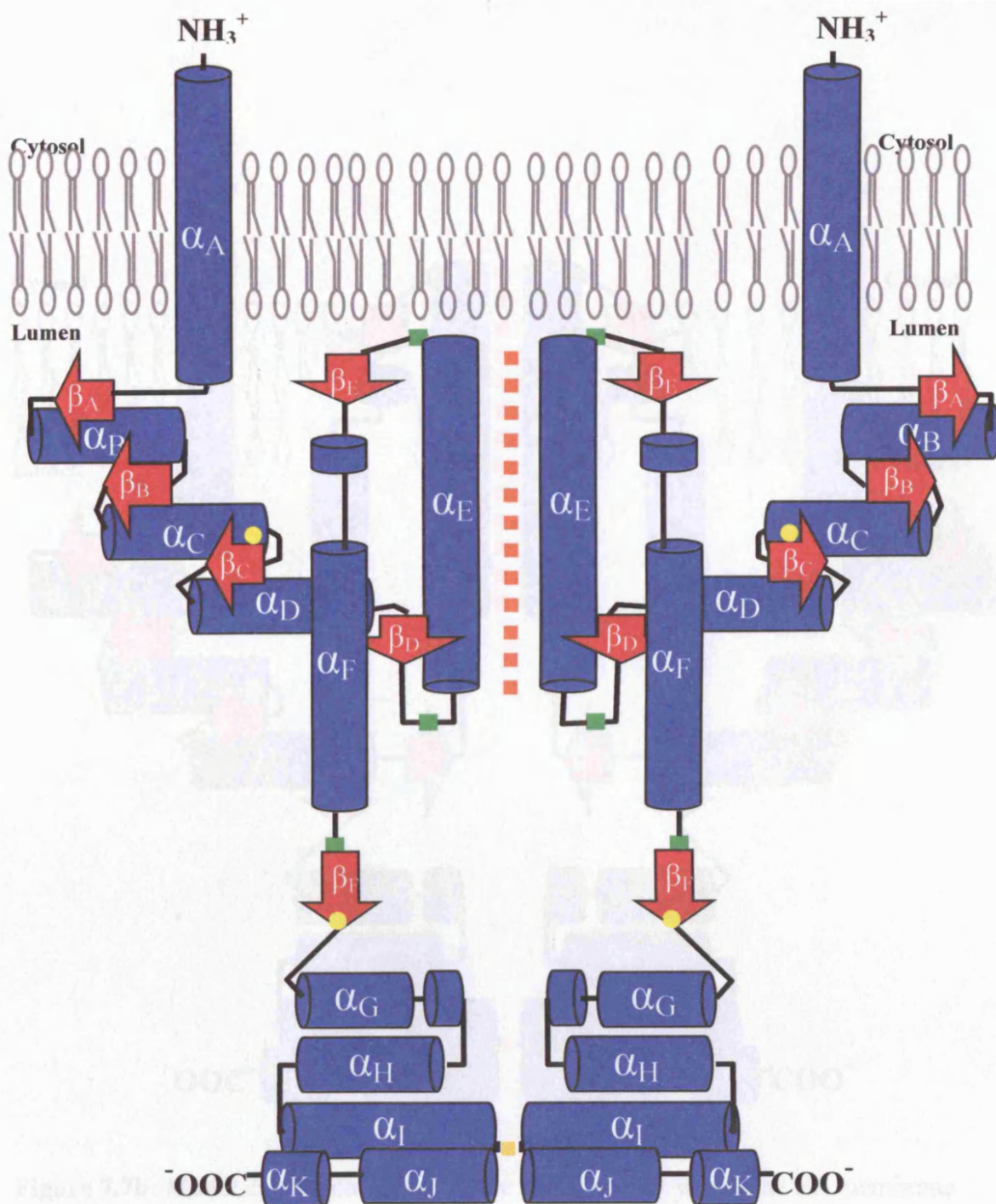







Figure 7.7a: Model 1 of secondary structure and topology within the ER membrane of human 11 β HSD1

 α -helix and  β -strand (each drawn to scale); — random coil
 (not drawn to scale); β_A - α_B - β_B - α_C = Rossmann fold (site of NADP(H) binding);
 β_E - α_F = Catalytic site; ■■■■ dimerization interface;  inter-molecular
 disulphide bond (human 11 β HSD1 only);  site of intra-molecular disulphide bond;
 consensus N-linked glycosylation sequence.

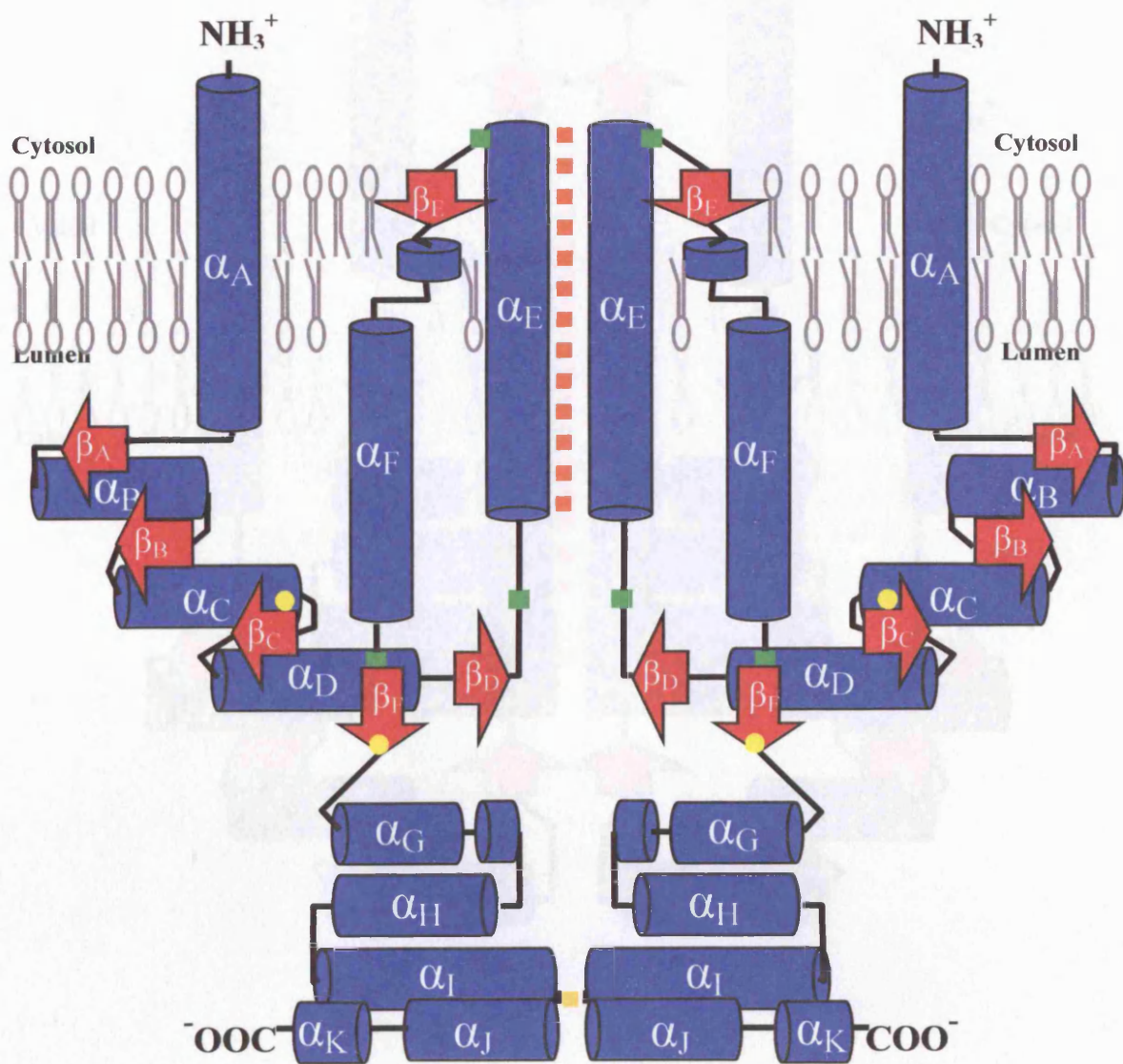
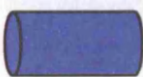




Figure 7.7b: Model 2 of secondary structure and topology within the ER membrane of human 11 β HSD1

 α -helix and  β -strand (each drawn to scale); — random coil (not drawn to scale); β_A - α_B - β_B - α_C = Rossmann fold (site of NADP(H) binding); β_E - α_F = Catalytic site; ■■■■ dimerization interface;  inter-molecular disulphide bond (human 11 β HSD1 only); ● site of intra-molecular disulphide bond; ■■■■ consensus N-linked glycosylation sequence.

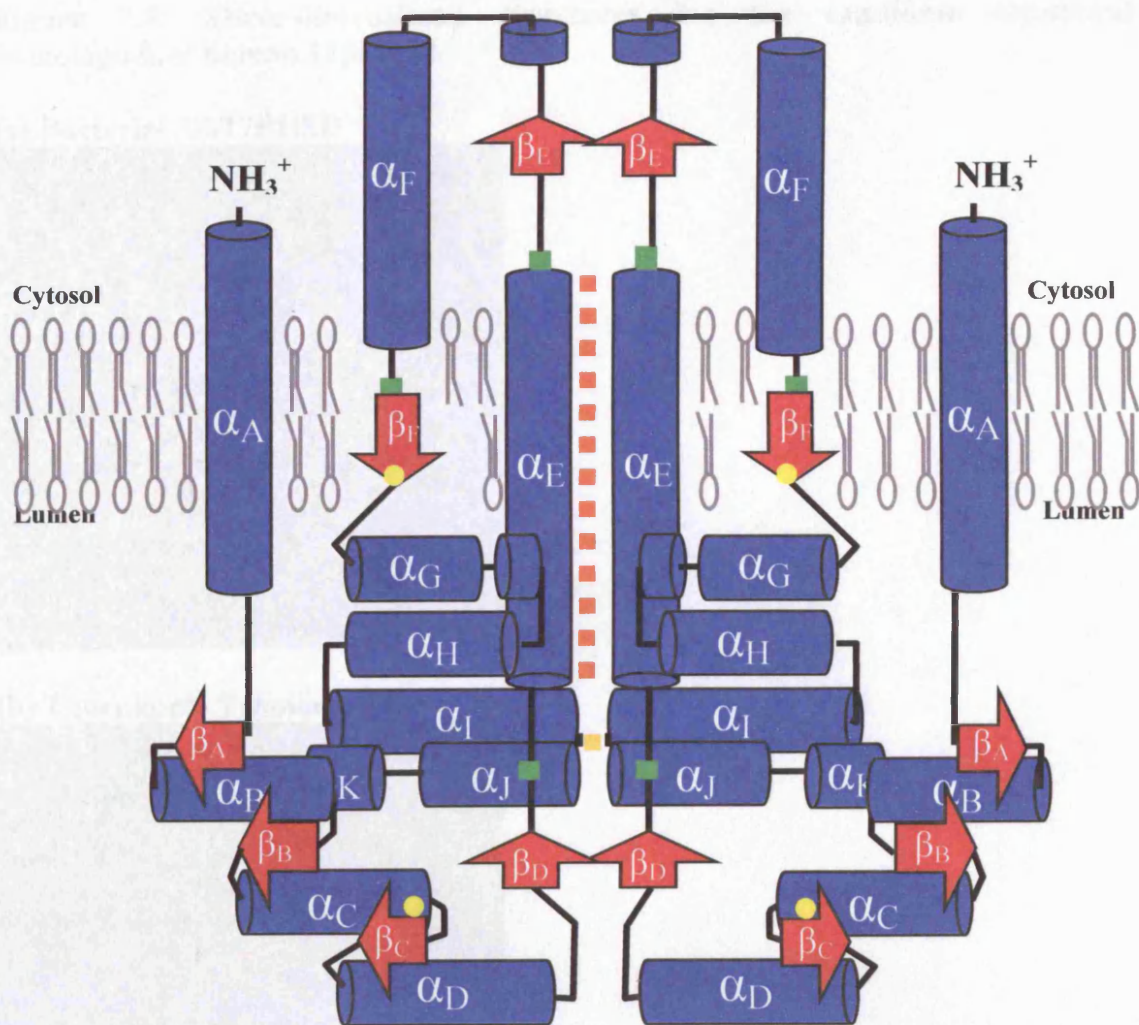


Figure 7.7c: Model 3 of secondary structure and topology within the ER membrane of human 11 β HSD1



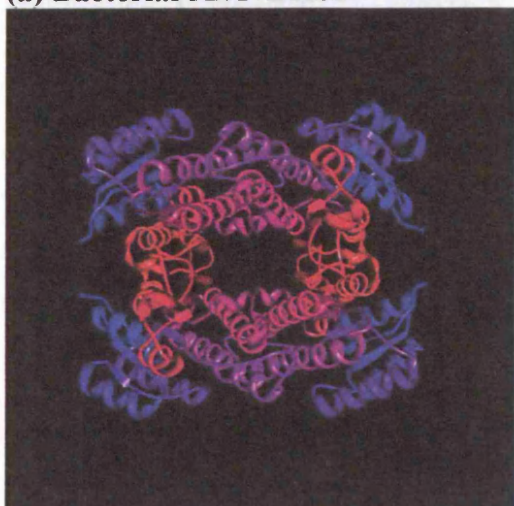
 α -helix and  β -strand (each drawn to scale); — random coil
 (not drawn to scale); β_A - α_B - β_B - α_C = Rossmann fold (site of NADP(H) binding);
 β_E - α_F = Catalytic site; ■ ■ ■ ■ dimerization interface; ■ inter-molecular
 disulphide bond (human 11 β HSD1 only); ● site of intra-molecular disulphide bond;
 consensus N-linked glycosylation sequence.

Figure 7.8: Three-dimensional structures for the candidate structural homologues of human 11 β HSD1

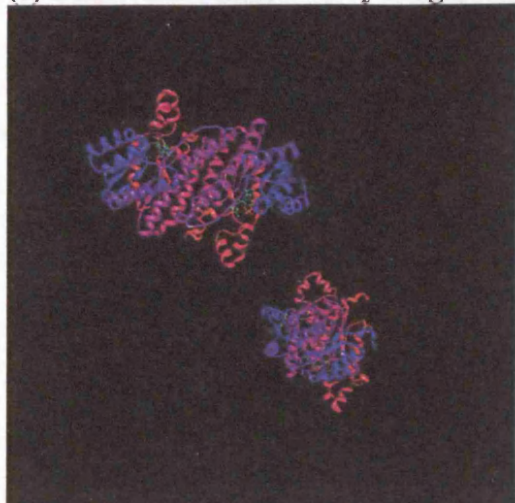
(a) Bacterial 3B/17BHSD



(b) Thornapple Tropionine Reductase 1



(c) Bacterial Glucose Dehydrogenase



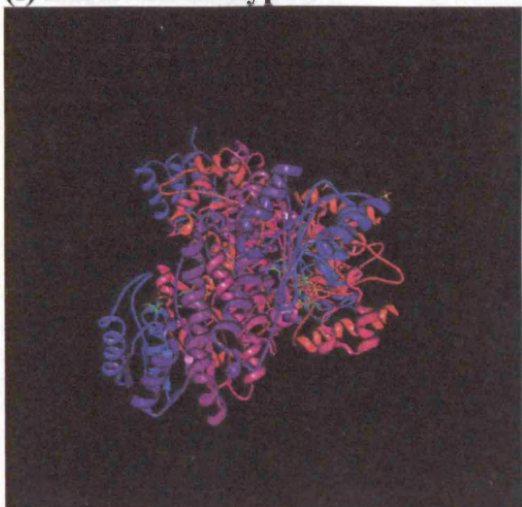
(d) Mouse Carbonyl Reductase



(e) Bacterial Sorbitol Dehydrogenase



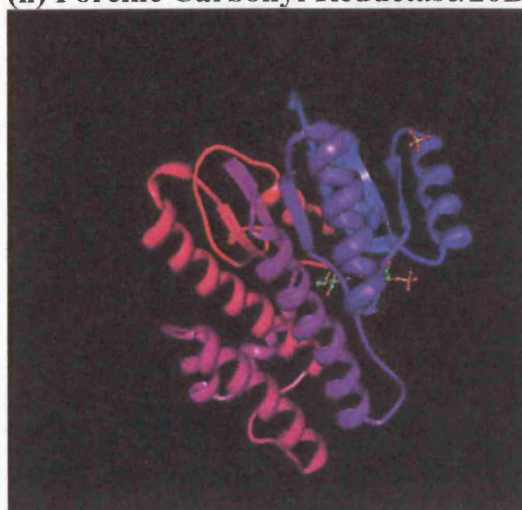
(f) Rat 17BHSD type IV



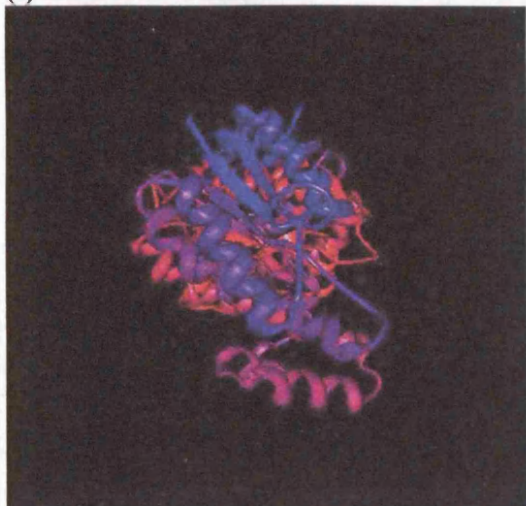
(g) Human 17BHSD type I



(h) Porcine Carbonyl Reductase/20BHSD



(i) Bacterial 3aHSD



(j) Rabbit 20aHSD



Table 7.1: Table outlining homology to human 11 β HSD1 primary sequence comprised of homology to whole sequence, N terminus before the catalytic region and C terminus following the catalytic region. Nd= not determined.

Species	N-terminus (prior to conserved YSASK catalytic sequence)		C-terminus (conserved YSASK catalytic sequence and beyond)		WHOLE PROTEIN	
	# same aa	% identity	# same aa	% identity	# same aa	% identity
Human	182	-	110	-	292	-
Monkey	173	95.1	92	83.6	265	90.8
Pig	154	84.6	84	76.4	238	81.5
Cow	148	81.3	82	74.5	230	78.8
Sheep	148	81.3	79	71.8	227	77.7
Cat	Nd	Nd	Nd	Nd	Nd	Nd
Rabbit	142	88.8	72	66.4	234	80.4
Hamster	151	83.0	79	71.8	230	78.8
Rat	158	86.8	74	67.3	232	79.5
Mouse	153	84.1	73	66.4	226	77.4

Table 7.2: Summary of essential criteria to assess the functionality of each proposed model for the topology of human 11 β HSD1. Where the given structural arrangement would prevent to enzyme being functional it is highlighted in red bold text. Green text represents a specific orientation of an amino acids or where a structural domain is consistent with published data.

	Model I	Model II	Model III
Number of putative transmembrane domains (TMDs)	1	3	3
Model exhibits the TMDs ¹ as predicted by:			
TMAP	-	-	YES
TopPred-KD ²	-	-	-
TopPred-GES ³	-	YES	-
MEMSAT2-V149 ⁴	YES	-	-
MEMSAT2-R/E149 ⁵	-	YES	-
TMHMM	YES	-	-
Position of specific amino-acids:			
Met ₁ (N-terminus) ⁶	Cytosol*	Cytosol*	Cytosol*
Cys ₇₈ ⁷	Lumen*	Lumen*	Lumen*
Asn ₁₂₃ ⁸	Lumen*	Lumen*	Lumen*
Asn ₁₆₂ ⁸	Lumen*	Cytosol	Cytosol
Asn ₂₀₇ ⁸	Lumen*	Lumen*	Membrane
Cys ₂₁₃ ⁷	Lumen*	Lumen*	Membrane
Cys ₂₇₂ ⁹	Lumen*	Lumen*	Lumen*
Lys ₂₉₂ (C-terminus) ⁶	Lumen*	Lumen*	Lumen*
Position of protein domains:			
Pyridine nucleotide cofactor binding domain	Lumen	Lumen	Lumen
Catalytic site (YxxxK)	Lumen	Membrane	Cytosol
Putative dimerisation interface (helix α E)	Lumen	Membrane	Membrane
Additional specifications:			
(1) Are the nucleotide cofactor binding domain and the catalytic site of the enzyme on the same side of the ER membrane?	YES*	YES*	<u>NO</u>
(2) Is Ser ₁₆₉ adjacent to Tyr ₁₈₃ such that these residues can form a catalytic reaction centre?	YES*	<u>NO</u>	YES*
(3) Can the putative dimerisation interface in helix α E form a 4-helix bundle with helices α F, α G and α H?	YES*	NO	NO

Table 7.3: Summary of mGenTHREADER tertiary structure homology

Protein	Organism (Common name)	PDB ID	EC	CATH	SCOP	Net Score	E value	Pairwise E	Solv. E	Align Score	A Len	D Len	T Len
3B / 17B-HSD	<i>Comamonas testosteroni</i> (Bacteria)	1hxx	1.1.1.51	3.40.50.720		0.972	0.00002	-1166.9	-15.0	697	250	253	292
Tropinone Reductase 1	<i>Datura stramonium</i> (Common thornapple)	1ae1	1.1.1.236	3.40.50.720		0.972	0.00002	-1139.7	-14.7	657	240	245	292
Glucose dehydrogenase	<i>Bacillus megaterium</i> (Bacteria)	1gco	1.1.1.47	3.40.50.720		0.972	0.00002	-1090.9	-17.8	783	254	261	292
Carbonyl reductase	<i>Mus musculus</i> (Mouse)	1cyd	1.1.1.184	3.40.50.720		0.972	0.00002	-1060.3	-14.4	642	240	242	292
Sorbitol dehydrogenase	<i>Rhodobacter sphaeriodes</i> (Bacteria)	1k2w	1.1.1.14	None	None	0.972	0.00002	-1058.0	-14.7	774	250	256	292
Estradiol 17B- dehydrogenase type 4	<i>Rattus norvegicus</i> (Rat)	1gz6	1.1.1.35	3.40.50.720		0.970	0.00002	-1047.7	-10.6	639	237	302	292
17BHSD type 1	<i>Homo sapiens</i> (Human)	1a27	1.1.1.62	3.40.50.720		0.968	0.00002	-950.7	-9.1	514	245	285	292

Carbonyl reductase / 20BHSD	<i>Sus scrofa</i> (Pig)	1hu4	1.1.1.53	3.40.50.720		0.965	0.00003	-957.5	-8.3	444	234	288	292
3aHSD	<i>Comamonas testosteroni</i> (Bacteria)	1fjh	1.1.1.50	3.40.50.720		0.960	0.00003	-797.2	2.2	410	205	236	292
20aHSD	<i>Oryctolagus cuniculus</i> (Rabbit)	1q5m	1.1.1.189	none	none	0.364	8.378	-534.7	-13.9	66	215	322	292

in the cytosol followed by a single transmembrane domain, and with the majority of the protein and the extreme C terminus localised to the ER lumen.

7.3.6 Protein fold predictions for the luminal domain of human 11 β HSD1

Based on the output of mGenTHREADER on 15 July 2004, 10 putative structures for the luminal domain of human 11 β HSD1 were isolated. These structures each had consistently high probabilities of structural similarity to 11 β HSD1 over 3 consecutive interrogations of the mGenTHREADER protein fold database (Table 7.3). The number of proteins identified with high fold homology to the luminal sequence of 11 β HSD1 is unusually high. The proteins identified include a number of members of the SCAD enzyme family including human 17 β HSD type 1, bacterial 3 β /17 β HSD, rat 17 β HSD type 4 and bacterial 3 α HSD (Figure 7.8). However, the protein rabbit 20 α HSD had a low fold homology with an E score of only 8.378 ($p > 0.05$ for homology to human 11 β HSD1).

7.4 Discussion

This study aimed to generate and evaluate alternative models for the topology and secondary/tertiary structures of 11 β HSD1. Combining relevant literature, sequence alignment data, hydrophobicity plots, topology predictions for 11 β HSD1 and secondary structure predictions (based on homology to other folds within the PsiPred and mGenTHREADER databases) 3 models for the possible tertiary structure and topology of 11 β HSD1 were generated. Each model was assessed by criteria that were known to be essential for 11 β HSD1 function, or that had been proven by *in*

vitro studies of 11 β HSD1 orientation in the ER membrane. Both Models 2 and 3 predicted structures that would conflict with published experimental findings. Specifically, model 2 would localise Asn₁₆₂ to the cytosol, however studies by Ozols [1995] have inferred that at least 1 Asn residue must be localised to the lumen of the ER for N-glycosylation. Also, in Model 2, key residues of Ser₁₆₉ and Tyr₁₈₃ would not lie within close enough proximity to form the catalytic triad required for functional enzyme activity as an oxidoreductase. Together, these findings render Model 2 an unlikely model for the topology and tertiary structure of 11 β HSD1 within the ER membrane. Model 3 localised both Asn₂₀₇ and Cys₂₁₃ to the membrane, and Asn₁₆₂ to the cytosol, conflicting with data by reported Ozols [1995]. Model 3 also localises the active site of 11 β HSD1 to the membrane, and the co-factor binding site to the lumen of the ER, therefore making Model 3 impossible for a functional enzyme.

Model 1 fulfils all of the expected criteria for functional 11 β HSD1. Published studies have hypothesised that 11 β HSD1 is a single transmembrane protein, with at least 2 sites for N-linked glycosylation, located in the lumen of ER [Ozols 1995]. Model 1 is in accordance with these predictions. Moreover, in Model 1 both the catalytic site and residues Ser₁₆₉ and Tyr₁₈₃ forming the catalytic reaction centre are of luminal orientation with Ser₁₆₉ able to adopt a position close to Tyr₁₈₃. The Rossman fold for NADP(H) binding is located within the lumen of the ER. The dimerisation interface is located within the lumen of the ER and the helices forming the dimer can form a four helix bundle as predicted by Tsignely and Baker [1995b] with α -helix_E, α -helix_F, α -helix_G and α -helix_H.

Although Model 1 upholds all structural requirements for 11 β HSD1 to be functional, there are still some unresolved problems with this Model. Firstly, exon 1 of the *HSD11B1* gene encodes the first 24 residues of 11 β HSD1, which almost certainly comprise the transmembrane domain of the protein. When translation is initiated at exon 2, the 11 β HSD1 protein is still targeted to the smooth ER and is membrane-associated. Loss of the N terminal amino acids, and transmembrane domain can be induced by site-directed mutagenesis, or physiologically, as is observed with 11 β HSD1B [Mercer *et al.*, 1993]. However, *in vitro* and *in silico* analysis have concluded that 11 β HSD1 is a single transmembrane protein. Having excluded the possibility of transmembrane domain membrane attachment, the association of N terminally truncated forms of 11 β HSD1 with the smooth ER membrane could reflect peripheral binding through myristoylation of 11 β HSD1. There are 5 predicted sites for myristoylation which could anchor 11 β HSD1 to the smooth ER membrane through one or more fatty acyl linkage (Figure 7.3).

In model 1, the consensus sequences for phosphorylation and the co-factor binding site both reside within the lumen of the ER. This poses problems for the transport of the pyridine nucleotides across the ER membrane. The lumen of the ER is known to support an oxidative environment [Hwang *et al.*, 1992] and is relatively impermeable to redox-active compounds including the pyridine nucleotides. It has been hypothesised that the oxidases and reductases that are anchored to the ER maintain a functional cooperativity to support this environment [Sawada and Carlson 1981; Kulkarni and Hodgson 1982; Draper *et al.*, 2003]. Hexose-6-phosphate

dehydrogenase (H6PDH) has been speculated to be a key enzyme in maintaining the homeostasis of the oxidative environment of the ER through the production of reducing equivalents of the pyridine nucleotides. H6PDH is the microsomal counterpart of the pentose phosphate pathway, utilising NADP^+ as a cofactor and functioning to catalyse the first 2 reactions in the pathway. The supply of substrate, glucose-6-phosphate, is ensured by the glucose-6-phosphate transporter [Clarke and Mason 2003]. The coupling of H6PDH and 11 β HSD1 was initially suggested based on cofactor requirements [Sawada and Carlson., 1991]. Recently it has been reported that co-segregating mutations in the genes encoding both 11 β HSD1 and H6PDH result in apparent cortisone reductase deficiency [Draper *et al.*, 2003]. Moreover, a recent study by Banhegyi *et al* [2004] has revealed direct cooperativity between the activity of H6PDH and 11 β HSD1 in rat liver microsomes. Specifically it has been proposed that H6PDH alters the redox state of NADP within the lumen of the ER highlighting a possible mechanism of nucleotide support for 11 β HSD1, and a possible mechanism for regulating 11 β HSD1 directionality, dependent upon the intra-luminal environment. Therefore an increase in glucose-6-phosphate into the cell will increase the conversion of glucose-6-phosphate to 6-phosphogluconate, which will increase the availability of NADPH, thus favouring cortisone reduction by 11 β HSD1 [Atanasov *et al.*, 2004].

The orientation of the kinase phosphorylation sites of 11 β HSD1 to the lumen of the smooth ER questions the ability of these kinase enzymes to directly phosphorylate the enzyme to regulate its activities. Numerous studies have shown that kinases and pharmacological stimulators and inhibitors of protein kinase activity can regulate the

activity of 11 β HSD1 (Table 1.1). However, these studies show the need for further investigation into the mechanisms by which 11 β HSD1 is regulated.

Although the two hydrophobic regions that flank the active site of 11 β HSD1 (Val₁₃₆ – Leu₁₅₈ and Ile₁₉₇ – Thr₂₂₀) are unlikely to form transmembrane domains, these hydrophobic regions could act as potential binding sites for hydrophobic molecules. Since such ligands would be binding to the hydrophobic core of the enzyme, adjacent to the active site of 11 β HSD1, this could provide a molecular mechanism whereby hydrophobic lipids could exert allosteric actions on enzyme activity. Such a mechanism could be hypothesised for the regulation of 11 β HSD1 activity via progesterone and/or prostaglandins.

In conclusion, this study reveals that the previously predicted topology for 11 β HSD1 within the ER is almost certainly correct. 11 β HSD1 does appear to be a single transmembrane glycoprotein, which forms homodimers and interacts with H6PDH. The N terminal amino acids are localised to the cytosol, followed by a single transmembrane domain and a large globular C terminal domain that contains hydrophobic regions, and could be linked to the luminal face of the ER membrane through fatty acyl linkages. Although not fully resolved, the mechanisms of regulation of enzyme activity include effects on the intra-luminal cofactor environment of the ER, based upon the co-operativity between 11 β HSD1 and H6PDH.

Chapter Eight: General discussion

Chapter 8: General Discussion

The goal of this thesis was to elucidate the cellular mechanisms controlling glucocorticoid metabolism by 11 β HSD in the human ovary. This goal was achieved by addressing 5 different questions:

- (i) What are the effects of compounds comprising human follicular fluid on 11 β HSD enzymes?
- (ii) What are the effects of local progesterone synthesis on the 11 β HSD enzyme(s) in human granulosa-lutein cells?
- (iii) How does local eicosanoid synthesis affect the 11 β HSD enzyme(s) in human granulosa-lutein cells?
- (iv) Do eicosanoids mediate the stimulation of ovarian 11 β HSD enzyme(s) by hCG and IL-1 β in human granulosa-lutein cells?
- (v) What are the structural topology, tertiary structure and mechanisms for regulation of 11 β HSD1 in the smooth ER?

The studies reported in this thesis utilised primary granulosa cell cultures for the study of cellular mechanisms controlling glucocorticoid metabolism by 11 β HSD in the ovary. The limitations of these *in vitro* granulosa cell cultures need to be noted. The granulosa cells were obtained from patients undergoing IVF-ET and so had been exposed to ovulatory doses of gonadotrophins, pre-disposing the cells to assume a luteal phenotype [Richards 1994]. The culture of isolated granulosa cells cannot take account of processes that occur with ovulation and corpus luteum formation, such as tissue remodelling and vascularisation of the follicle. However, primary granulosa

cell cultures, as compared with granulosa cell lines, do retain functional responses and the ability to maintain steroidogenesis with the pattern of steroid synthesis *in vitro* resembling the hormonal profile *in vivo*.

Results reported in Chapter 3 of this thesis have demonstrated that there are hydrophilic stimulators and hydrophobic inhibitors of NADP(H)-dependent 11 β HSD activities in hFF. Under the conditions used in this study, these compounds have no effect of NAD⁺-dependent cortisol oxidation. Moreover, the compounds present in hFF exert their effects on the NADP(H) dependent 11 β HSD1 activities without affecting protein expression. Although the identity of the compounds remains unclear, the predominant hydrophobic inhibitor of 11 β HSD1 activities is not a known prostaglandin, steroid or cholesterol. Moreover, studies have shown that GALF is not a candidate for either the hydrophilic or hydrophobic modulators of 11 β HSD1 activity in human FF. At present, based on biophysical properties, oxysterols and/or bile acids are the strongest candidates for the hydrophobic inhibitor(s) of 11 β HSD human FF.

Limitations of this study were that concentration-dependent effects of the modulatory compounds in human FF on 11 β HSD1 activity were not determined. Also, the kinetic properties of the modulatory hydrophobic components of human FF were not determined. A kinetic study would enable a fuller understanding of the type of inhibition that the modulatory components exert on 11 β HSD1 activity.

One potential mechanism by which the hydrophobic components of human FF could inhibit the bi-directional activities of 11 β HSD1 is by binding to the active site of the enzyme. Were this to be the case, then the inhibition of enzyme activity would be competitive in nature. Hence, this possibility could be assessed by investigating whether the hydrophobic components of human FF can increase the K_m of 11 β HSD1 for cortisol or cortisone, ideally in a cell-free system such as liver microsomes. The studies reported in Chapter 7 raised the possibility that hydrophobic compounds in human FF might inhibit 11 β HSD1 by allosteric actions. This could involve binding to the hydrophobic regions which flank the active site of the enzyme.

In the studies reported in Chapter 4, inhibition of endogenous progesterone production increased both the oxidative and reductive activities of 11 β HSD1 in human granulosa-lutein cells, without affecting the expression of 11 β HSD1 protein. Interestingly although progesterone appeared to inhibit basal 11 β HSD activities, it was notable in that in the studies reported in Chapter 6, inhibition of progesterone using AG has little impact on the stimulation of 11 β HSD stimulated activities by hCG. Intuitively it would have been expected that hCG would have stimulated progesterone production which would have attenuated any stimulation of 11 β HSD activities. If this were so, co-treatment with AG would be expected to enhance the stimulation of enzyme activities by hCG. However this was not found to be the case, suggesting that elevation of progesterone has no bearing on the endocrine stimulation of 11 β HSD-mediated glucocorticoid metabolism in human granulosa-lutein cells.

Limitations of this study were that AG (an inhibitor of P450_{csc}) was used as a pharmacological tool to inhibit progesterone production. AG is known to non-specifically inhibit other P450 enzymes of steroidogenesis such as aromatase [Santen and Misbin, 1981] and therefore could non-specifically affect the activity of 11 β HSD. However, co-treatment with AG has been shown to stimulate the activity of 11 β HSD, which could be due to AG inhibiting the synthesis of a regulator of 11 β HSD, rather than through a direct effect of AG itself on the activity of the enzyme.

AG directly inhibits the conversion of cholesterol to pregnenolone (the rate limiting step of steroidogenesis). Pregnenolone is subsequently converted by 3 β HSD to progesterone. Therefore the inhibition of P450_{csc} not only inhibits progesterone synthesis, but also pregnenolone synthesis. Thus pregnenolone could also be exerting an inhibitory effect on 11 β HSD activity that is removed by the presence of AG. To investigate this, pregnenolone concentrations could be determined in the presence and absence of AG, to confirm that AG does inhibit pregnenolone production. Also, pregnenolone and progesterone replacement experiments could be conducted in the presence of AG, although there are limitations to these experiments as discussed in section 4.4. Moreover, due to progesterone being the major secretory product of the CL it is likely that progesterone is the predominant inhibitor of 11 β HSD activity in hGL cells.

The cellular mechanisms by which progesterone could regulate the activities of 11 β HSD have been speculated on in Chapter 4. The simplest explanation is that

progesterone could bind to the active site of 11 β HSD1, thus competing with cortisol and/or cortisone. Alternatively the regulatory mechanisms could involve allosteric binding of progesterone to hydrophobic interfaces in the structure of 11 β HSD1. The studies reported in Chapter 7 indicated that such regions exist on both sides of the active site of 11 β HSD1.

Studies reported in Chapter 5 have shown that inhibition of endogenous prostaglandin synthesis using MA and NS-398 decreased 11 β HSD activities without affecting the expression of 11 β HSD1 protein. These data suggest a role for endogenous prostaglandin synthesis in elevating the basal activities of 11 β HSD in human granulosa-lutein cells. However, addition of exogenous prostaglandins did not overcome the inhibition of 11 β HSD activity induced by the addition of MA and NS-398, calling into question the interpretation of the initial finding. As conceded in Chapter 5 an alternative explanation of these findings would be that other eicosanoids (e.g. leukotrienes) could have been non-specifically inhibited by NS-398 and/or MA. Alternatively the inhibition of prostaglandin synthesis may have re-directed the flux of AA metabolism towards the epoxygenase and lipoxygenase pathways where products of these pathways may have a negative effect on 11 β HSD enzyme activities. Findings in Chapter 5 also raise the possibility that exogenously administered prostaglandins may not exert the same actions as prostaglandins synthesised by PGHS2 within the lumen of the smooth ER or within the nucleus of the cell.

The cellular mechanisms by which eicosanoids could regulate the activities of 11 β HSD could be through allosteric regulation of 11 β HSD. PGHS1 and PGHS2 are localised to the lumen of the ER and to the luminal side of the nuclear envelope. Studies conducted in Chapter 7 are consistent with previous suggestions that the active site of 11 β HSD1 is localised to the lumen of the smooth ER, and have found the active site of 11 β HSD1 to be flanked by hydrophobic regions. Therefore prostaglandins generated in the lumen of the ER could act (without being secreted from the cell) allosterically within the lumen of the ER to increase the activities of 11 β HSD. Prostaglandins could also be acting in the more classical manner through paracrine and autocrine mechanisms via secretion from cells, and through the binding to cells surface GPCRs, activating second messenger signalling cascades and protein kinases. However, studies in Chapter 7 of this thesis question the ability of protein kinases to phosphorylate 11 β HSD1 due to orientation of the enzyme, with the consensus phosphorylation sequences present in the lumen of the ER. Also, the addition of exogenous PGs, which would be expected to act through a second messenger-protein kinase signalling cascade, does not overcome the inhibition of 11 β HSD1 observed when intracellular prostaglandin synthesis is inhibited using MA and NS-398.

A recent publication by Rae *et al.* [2004] has revealed a potential link between the expression of PGHS2 mRNA and the expression of 11 β HSD1 mRNA when hOSE cells are stimulated by IL-1 α . After 6 hours exposure to IL-1 α , PGHS2 mRNA expression was elevated, and after 12 hours exposure to IL-1 α , an increase in 11 β HSD1 mRNA was also observed. The difference in time could be due to IL-1 α

inducing an increase in 11 β HSD1 mRNA expression through increasing prostaglandin generation. These data are consistent with a model in which prostaglandins mediate the stimulation of 11 β HSD1 activity and/or expression by hCG and IL-1. A model whereby the effects of hCG and IL-1 β on prostaglandin synthesis and 11 β HSD1 activities are causally linked (rather than independent) is logical. Were hCG and/or IL-1 β to simultaneously stimulate prostaglandin synthesis and the regeneration of cortisol by 11 β HSD1, cortisol may exert its anti-inflammatory actions too rapidly, before prostaglandins have fulfilled their pro-inflammatory roles required for ovulation. A model in which the elevation of prostaglandins has to precede the stimulation of 11 β HSD1 would allow the prostaglandins to up-regulate MMPs and affect the vascularisation of the follicle before local glucocorticoid synthesis limits further eicosanoid generation.

Studies reported in Chapter 6 have shown that MA and NS-398 can prevent hCG and IL-1 β from stimulating 11 β HSD activities without affecting 11 β HSD1 protein expression. These data could imply a mediatory role for prostaglandins in the stimulation of 11 β HSD activities by hCG and IL-1 β . However, it is also possible that, as previously discussed for Chapters 5 and 6, the inhibition of prostaglandin synthesis has favoured the generation of epoxygenase and/or lipxygenase products that inhibit the activities of 11 β HSD in human granulosa-lutein cells.

To explore the possible involvement of other eicosanoid products in hCG- and IL-1 β - stimulated 11 β HSD activities, inhibitors of the lipxygenase pathway such as NDGA (which inhibits the 5-, 12-, and 15-lipxygenase pathways) could be used. If

co-treatment with NDGA inhibits the stimulation of 11 β HSD activities by hCG and IL-1 β , it would be suggestive of lipoxygenase products playing a mediatory role to increase 11 β HSD activities.

Since exogenous prostaglandins do not affect 11 β HSD enzyme activities (Chapter 5) and the potential kinase regulatory sites of 11 β HSD1 are orientated to the lumen of the smooth ER (Chapter 7), it seems likely that the cellular mechanisms by which prostaglandins can regulate 11 β HSD activities involve allosteric regulation. To investigate the possibility that prostaglandins are allosterically regulating 11 β HSD, studies using liver microsomes could be carried out. The microsomes could be used as a source of 11 β HSD1 enzyme, and the direct effects of prostaglandins could be assessed using radiometric conversion assays. A caveat to this study is that if no stimulation is seen, then this could be due to accessibility problems, as prostaglandins are normally generated on the luminal side of the ER (and nucleus) by PGHS, whereas exogenous prostaglandins would be added on the cytosolic face of the microsomes. Therefore the addition of exogenous prostaglandins might not be able to penetrate the microsomal membrane, as may be the case with the intact human granulosa-lutein cell system. Alternatively, binding studies could be carried out using liver microsomes or HEK-293 cells stably transfected with 11 β HSD1. Tritiated prostaglandins could be added to the system, and bound versus free prostaglandins could be quantified. A control to this experiment would be to repeat the same experimental design with mock transfected HEK-293 cells.

More sophisticated techniques could be used to further dissect whether prostaglandins can allosterically regulate 11 β HSD. For example, binding studies could also be carried out using affinity chromatography. Affinity columns could be generated with prostaglandins and purified 11 β HSD1 protein could be loaded onto the column to assess binding of the 11 β HSD1 to the immobilised prostaglandins. X-ray crystallography could also be used to determine whether prostaglandins interact with 11 β HSD1. Crystals could be formed in the absence and presence of prostaglandins and a conformational change in 11 β HSD1 structure would be observed if the mechanism of regulation was allosteric.

Further studies could be carried out to elucidate the mechanisms by which hCG and IL-1 β stimulate 11 β HSD1 activities. The signalling pathways through which the hormones are mediating the response could be dissected using pharmacological tools such as inhibitors and receptor agonists and antagonists and kinase activators/inhibitors. The use of such agents would enable each step of signalling pathways to be investigated starting from receptor, to second messenger generation, protein kinase activation, and initiation/inhibition of transcription/translation.

A mechanism of emerging importance is that of co-factor balance regulating the predominant direction in which the enzyme acts. As noted earlier, co-operativity is thought to exist between 11 β HSD1 and H6PDH within the lumen of the ER. H6PDH catalyses the conversion of hexose-6-phosphate to 6-phosphogluconate which requires NADP⁺ conversion to NADPH. Thus altering the co-factor balance of the lumen of the ER in favour of 11KSR activity of enzyme [Atanasov *et al.*, 2004;

Banhegyi *et al.*, 2004]. However, it is important to acknowledge that human granulosa-lutein cells are steroidogenic, and as such, have a high demand for NADPH in the lumen of the smooth ER. This reductant co-factor is required for the activities of steroidogenic cytochrome P450 and HSD enzymes such as CYP17, CYP19, and 17 β HSD1. The combined activities of these enzymes will result in the oxidation of NADPH to NADP⁺, hence, increasing the NADP⁺:NADPH ratio within the lumen of the smooth ER (Figure 8.1). The direction of the enzyme has been questioned in steroidogenic gonadal cells with a predominant oxidative direction of 11 β HSD1 reported in both Leydig cells [Gao *et al.*, 1997s; Ge *et al.*, 1997; Ge and Hardy 2000] and human granulosa-lutein cells [Michael *et al.*, 1997], suggesting that the co-factor balance may be different in steroidogenic cells. As suggested in Figure 8.1, it seems likely that within steroidogenic cells, the activity of steroidogenic enzymes increases the availability of NADP⁺ to favour the oxidative activity of 11 β HSD1, in much the same way as H6PDH can generate NADPH to favour the 11KSR activity.

When comparing data obtained from each Chapter of this thesis, it is noteworthy that each of the regulatory compounds differentially regulate the activities of 11 β HSD1. The regulatory components of human FF appear to regulate both the 11 β BDH and the 11KSR activities of 11 β HSD1 with equal affinities. Inhibition of progesterone and prostaglandins production appears to predominantly regulate the 11KSR activity of 11 β HSD1. Whereas, hCG and IL-1 β appear to predominantly increase the 11 β BDH

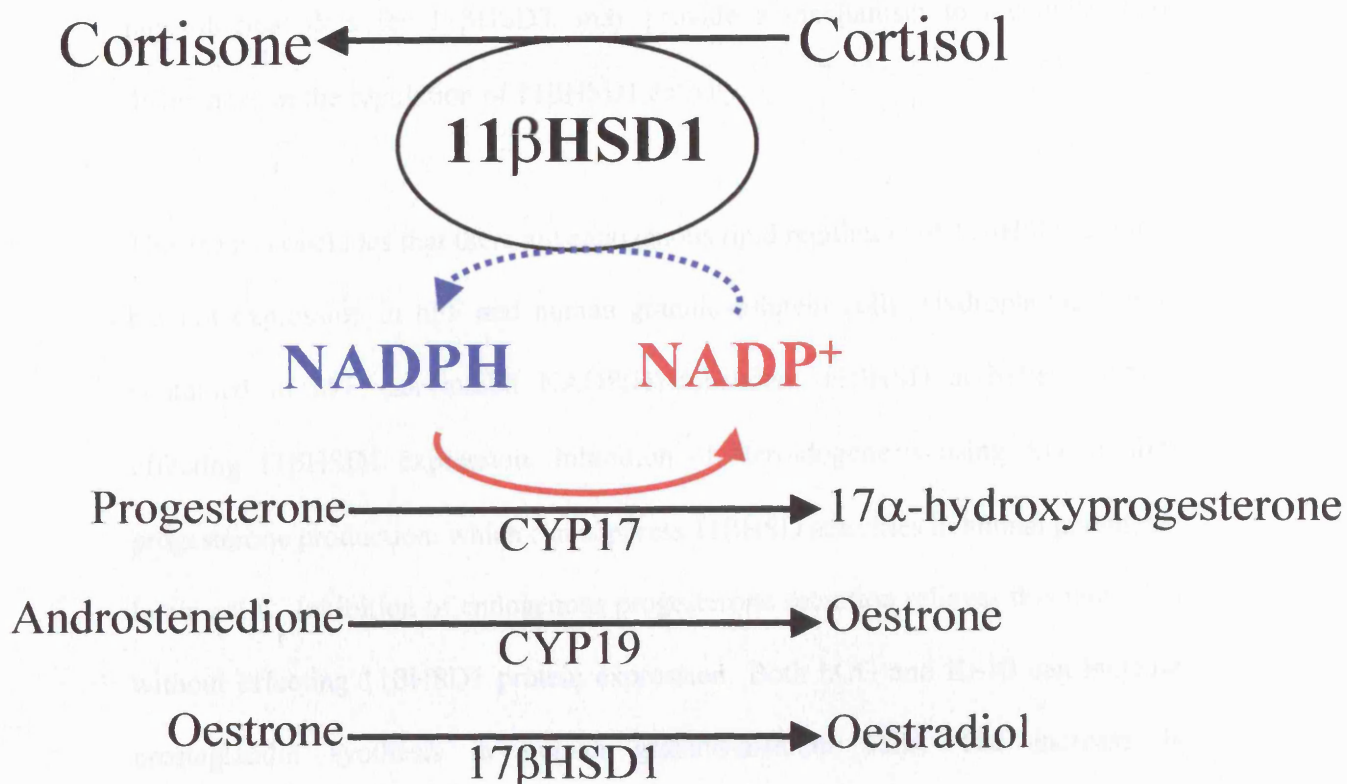


Figure 8.1: Steroidogenic pathways utilising NADP(H) in human granulosa-lutein cells

activity of 11 β HSD1. These data remain un-reconcilable, and perhaps question whether the 11 β DH and the 11KSR activities reside in a single protein. However, the recent deposition of the sequence for a third isoform of 11 β HSD in the NCBI database may resolve this quandary, and on publication of the biochemical, and physiological data for 11 β HSD3, may provide a mechanism to reconcile these differences in the regulation of 11 β HSD1 activity.

This thesis concludes that there are endogenous lipid regulators of 11 β HSD activities but not expression in hFF and human granulosa-lutein cells. Hydrophobic factors contained in hFF can inhibit NADP(H)-dependent 11 β HSD activities, without effecting 11 β HSD1 expression. Inhibition of steroidogenesis using AG, inhibits progesterone production, which can suppress 11 β HSD activities in human granulosa-lutein cells. Inhibition of endogenous progesterone secretion relieves this inhibition without effecting 11 β HSD1 protein expression. Both hCG and IL-1 β can increase prostaglandin synthesis in human granulosa-lutein cells. The increase in prostaglandin synthesis may mediate an increase in 11 β HSD1 activities, but not protein expression. This suggests a possible positive feedback mechanism by which inflammatory effectors of ovulation such as cytokines and LH, through up-regulating PGHS2 and prostaglandin production can increase cortisol production, which may then limit the inflammatory event of ovulation (Figure 8.2).

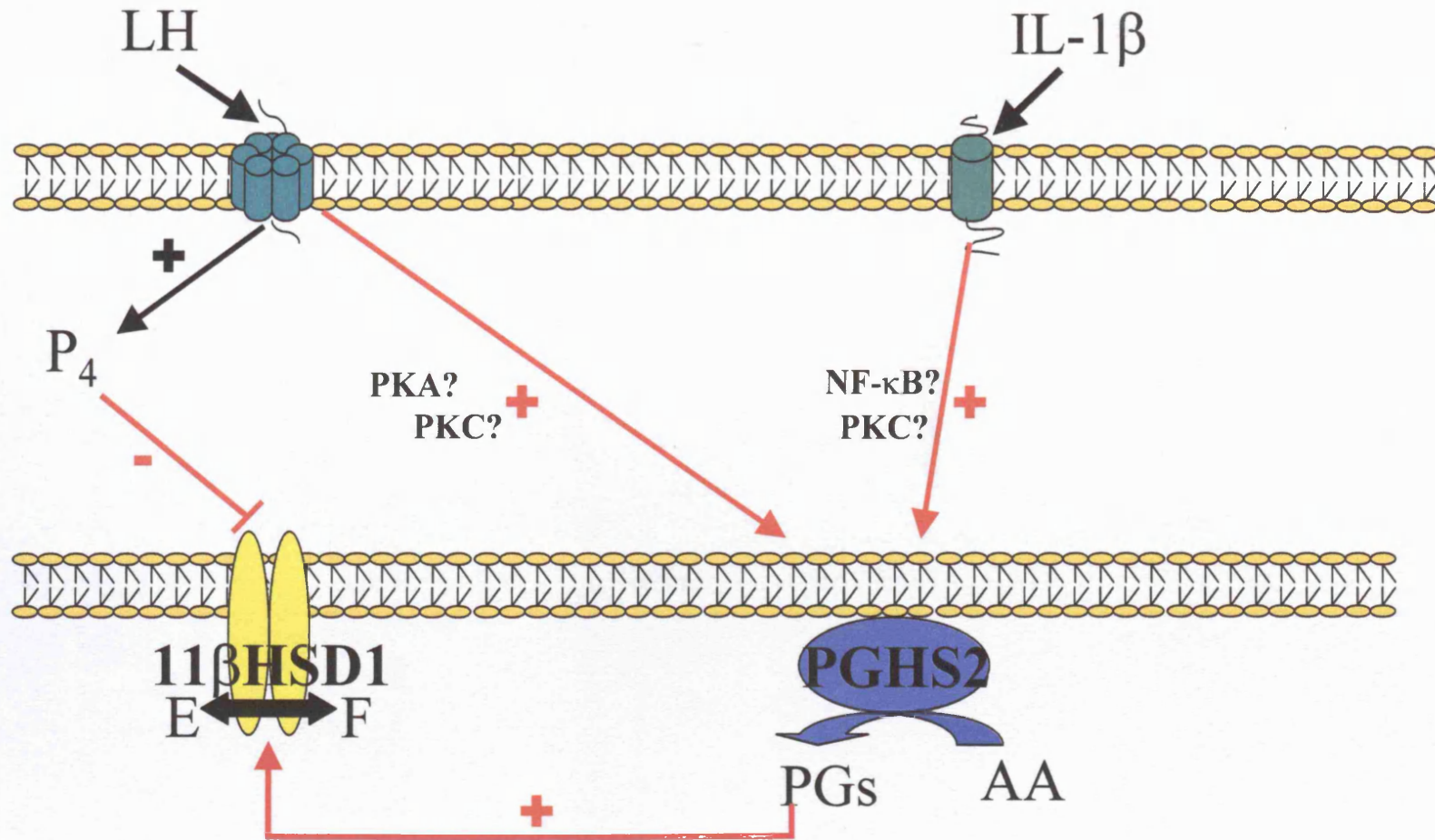


Figure 8.2 Proposed mechanism by which PGs and progesterone regulate 11HSD activities. Red arrows= thesis findings, Black arrows= hypothesised mechanisms

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Appendix I

Appendix I

A. 60% Percoll Density Gradient Solution

Reagent/Solution	Volume per 15ml
Percoll	9ml
PBS	5ml
1.5M NaCl (filter sterilised)	1ml

B. PAS-Gelatin Buffer for Progesterone RIA at pH 7.0

Reagent	Mass (g/l)
NaH ₂ PO ₄ .2H ₂ O	0.6
Na ₂ HPO ₄ .12H ₂ O	2.2
NaCl	9.0
NaN ₃	1.0
Gelatin	1.0

C. Tris Buffer for Prostaglandin RIA at pH 7.4

Reagent	Mass (g/l)
Gelatin	1
Tris HCl	6.61
NaN ₃	0.2
Tris Base	0.97

D. Lysis Buffer for Rat Kidney Homogenate assay at pH 7.4

Reagent	Mass (g/l)
Trizma Base	0.6
MgCl ₂	0.3
EDTA	0.6

E. Lysis Buffer for Protein preparation for SDS-PAGE gel electrophoresis

Reagent	Quantity
76.5mM Tris pH 6.8	83ml
SDS	2g
Glycerol	10ml

F. Transfer Buffer for Western Blot X10

Reagent
390mM glycine
480mM Tris
0.375% (w/v) SDS

G. Transfer Buffer for Western Blot X1

Reagent	Quantity
Transfer buffer X10	100ml
Methanol	200ml
dH ₂ O	700ml

H. TBST (Tris buffered saline-Tween) for Western Blot pH 6.7 X10

Reagent
500mM Tris
1.5M NaCl
0.2% Tween

I. Samples Buffer for SDS-PAGE electrophoresis

Reagent	Quantity
dH ₂ O	7.25ml
0.5M Tris HCl (pH 6.8)	1.25ml
Glycerol	1ml
β-mercaptoethanol	0.5ml
SDS	0.2g
Bromophenol blue	0.01g

J. 10% resolving gel for reducing SDS-PAGE gel

Reagent	Quantity
BDH Ultrapure water	19.8ml
30% Polyacrylamide	16.7ml
Tris-HCl (pH 8.4)	12.5ml
10% SDS (w/v)	500μl
10% (NH ₄) ₂ S ₂ O ₈	500μl
TEMED	20μl

K. 4% stacking gel for reducing SDS-PAGE gel

Reagent	Quantity
BDH Ultrapure water	12.2ml
30% Polyacrylamide	2.6ml
Tris-HCl (pH 6.8)	5ml
10% SDS (w/v)	200μl
10% (NH ₄) ₂ S ₂ O ₈	125μl
TEMED	25μl

L. Stripping buffer for removal of bound antibody from Western blots

Reagent	Quantity
β -mercaptoethanol	700 μ l
SDS	2g
62.5mM Tris-HCl (pH 6.7)	100ml

Appendix II

Appendix II

Type 1 11βHSD : PROTEIN sequences

Human (292 aa)

MAFMKKYLLP	ILGLFMAYYY	YSANEEFRPE	MLQGKKVIVT
GASKGIGREM	AYHLAKMGAG	VVVTARSKET	LQKVVSCHLE
LGAASAHYIA	GTMEDMTFAE	QFVAQAGKLM	GGLDMLILNH
ITNTSLNLFH	DDIHHVRKSM	EVNFLSYVVL	TVAALPMLKQ
SNGSIVVVSS	LAGKVAYPMV	AAYSASKFAL	DGFFSSIRKE
YSVSRVNVSI	TLCVLGLIDT	ETAMKAVSGI	VHMQAAPKEE
CALEIIKGGG	LRQEEVYYDS	SLWTTLLIRN	PCRKILEFLY
	STSYNMDRFI	NK	

MAFMKKYLLPILGLFMAYYYYSANEEFRPEMLQGKKVIVTGASKGIGREMAYHLAK
MGAHVVVTARSKETLQKVVSCHLELGAASAHYIAGTMEDMTFAEQFVAQAGKLMGG
LDMLILNHITNTSLNLFHDDIHHVRKSMEVNFLSYVVLTVAAALPMLKQSNGSIVVV
SSLAGKVAYPMVAAYSASKFALDGFFSSIRKEYSVSRVNVSIITLCVLGLIDTETAM
KAVSGIVHMQAAPKEECALEIIKGGALRQEEVYYDSSLWTTLLIRNPCRKILEFLY
STSYNMDRFINK

Squirrel Monkey (291 aa)

MAFMKTHLLP	ILGLFMAYYY	YSAYEEFRPE	MLQGKKVIVT
GASKGIGREM	AYHLAKMGAG	VVVTARSKET	LQKVVSCHLE
LGAASAHYIA	GTMEDMTFAE	QFVAQAGKLM	GGLDMLILNH
ITNTSLNFFH	DDIHHVRKSM	EVNFLSYVVL	TVAAMPMLKQ
SNGSIVIVSS	VAGKVAYPMI	SAYSASKFAL	YGFFSSIRKE
YLMSEVNVSI	TLCVLGLIDT	DTAMKAVSGI	IKMQAARKEE
CALEIIKGGV	LRQEEVYYDR	SLWTTLLLRN	PSRKILEFLR
STSYSTDGLI	N		

MAFMKTHLLPILGLFMAYYYYSAYEEFRPEMLQGKKVIVTGASKGIGREMAYHLAK
MGAHVVVTARSKETLQKVVSCHLELGAASAHYIAGTMEDMTFAEQFVAQAGKLMGG
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SSVAGKVAYPMISAYSASKFALYGFFSSIRKEYLMSEVNVSIITLCVLGLIDTDTAM
KAVSGI IKMQAARKEECALEIIKGGVLRQEEVYYDRSLWTTLLLRNPSRKILEFLR
STSYSTDGLIN

Pig (292 aa)

MAFMKKYLLP	ILGIFLAYYY	YSANEEFRPE	MLRGKKVIVT
GASKGIGREM	AYHLARMGAH	VVVTARSEET	LKKVVSHSLE
LGASSAHYVA	GTMEDMTFAE	QFVAKAGKLL	GGLDMLILNH
ITQAFMTSVY	DDIHLVRRSM	EVNFLSYVVL	SVAALPMLKQ
SNGSIVVVSS	QAGKMANPLV	APYSASKFAL	DGFFSSITKE
YSVTKVNVSI	TLCILGLIDT	DTAMTAVAGI	LVNQPSPKKE
CALEIIKGAA	LRQEEVYYDS	SILTSLLLEN	PGRKILEFLS
LRHYNMERFT NN			

MAFMKKYLLPILGIFLAYYYYSANEEFRPEMLRGKKVIVTGASKGIGREMAYHLAR
 MGAHVVTARSEETLKKVVSHSLELGASSAHYVAGTMEDMTFAEQFVAKAGKLLGG
 LDMLILNHITQAFMTSVYDDIHLVRRSMEVNFLSYVVLVAALPMLKQSNGSIVVV
 SSQAGKMANPLVAPYSASKFALDGFFSSITKEYSVTKVNVSITLCILGLIDTDTAM
 TAVAGILNVQPSPKKECALEIIKGAALRQEEVYYDSSILTSLLLENPGRKILEFLS
 LRHYNMERFTNN

Cow (292 aa)

MAFMKKYLLP	ILGIFLAYYY	YSANEEFRPE	MLRGKRVIVT
GASKGIGREM	AYHLARMGAH	VVVTARSEES	LKKVVSRCLE
LGAASAHYVA	GTMENMTFAE	QFVAKAGELV	GGLDMLILNH
IHYTPLGVFS	NDIHLRLRTL	EVNLLSYVVL	STAALPMLKQ
TNGSIVVVSS	IAGKIACPLV	AAYSASKFAL	DGFFSSLRME
YEATKVNVS	TLCILGLIDT	DTAMKAVAGI	FNAKASPKEE
CALEIIKGGT	LRQDEVYYDS	SILTPLLRLN	PGRKIMEFFF
LKKYNMERFI NN			

MAFMKKYLLPILGIFLAYYYYSANEEFRPEMLRGKRVIVTGASKGIGREMAYHLAR
 MGAHVVTARSEESLKKVVSRCLELGAASAHYVAGTMENMTFAEQFVAKAGELVGG
 LDMLILNHIHYTPLGVFSNDIHLRLRTLEVNLLSYVVLSTAALPMLKQTNGSIVVV
 SSIAGKIACPLVAAYSASKFALDGFFSSLRMEYEATKVNVSITLCILGLIDTDTAM
 KAVAGIFNAKASPKECALEIIKGGTLRQDEVYYDSSILTPLLRLNPGRKIMEFFF
 LKKYNMERFINN

Sheep (290 aa)

MAFMKKYLLP	ILGLFLAYYY	YSANEEFRPE	MLRGKRVIVT
GASKGIGREM	AYHLARMGAH	VVVTARSEES	LKKVVSRCLE
LGAASAHYVA	GTMENMTFAE	QFVAKAGELV	GGLDMLILNH
INYTPLRVFS	NDIHLRRSL	EVNLLSYVVL	STAALPMLKQ
TSGSIVVVSS	VAGKIACPLA	AAYSASKFAL	DGFFSSLRTE
YEATKVNVS	TLCILGLIDT	DTAMKAVAGI	YNAEASPKEE
LEIIKGGALR	QDEVYYDNSI	LTSLLLKNPG	RKIMEFLSLK
KYNMERFINN			

MAFMKKYLLPILGLFLAYYYYSANEEFRPEMLRGKRVIVTGASKGIGREMAYHLAR
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 KYNMERFINN

Cat – PARTIAL (Partial = 193 aa)

.....VIVT	GASKGIGEQM	
AYHLAKMGAH	VVVTARSKEN	LKKIVSHCME
LGAASAHYIA	GTMENMTFAE	QFVAKAGKLM
GGLDMLILNH	ITNTSMNLFS	GDIHIVRRSM
EVNFLSYVVL	SATALPMLKQ	SNGSIVVVSS
KAGKMASPLI	APYSASKFAL	DGFFSSIRME
HSVAKINVSI	TLCILGLINT	DTAMNAISG.

...VIVTGASKGIGEQMAYHLAKMGAHVVVTARSKENLKKIVSHCMELGAASAHYI
 AGTMENMTFAEQFVAKAGKLMGGLDMLILNHITNTSMNLFSGDIHIVRRSMENVFL
 SYVVLSTALPMLKQSNGSIVVVSSKAGKMASPLIAPYSASKFALDGFFSSIRMEH
 SVAKINVSITLCILGLINTDTAMNAISG...

Rabbit (291 aa)

AFMKKYLLPL	LGLFLAYYYY	SANEEFRPEM	LQGKKVIVTG
ASKGIGKEIA	FHLAKMGAHV	VVTARSKETL	QEVVAHCLKL
GAASAHYIAG	TMEDMTFAEQ	FVAKAGKLMG	GLDMLILNHI
TNASLMFFNN	DIHHVRKEME	VNFLSYVVL	VAALPMLKQS
NGSIVVVSSL	AGKIAHPLIA	PYSASKFALD	GFFSAIRKEH
ALTNVNVSIT	LCVLGLIDTD	TAMKEVSGKI	DMKAAPKEEC
ALEIIKGGAL	RQDEVYYGNL	QWTPLLLGNP	GKRLIEFLHL
RKFDISKLVN N			

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DMLILNHITNASLMFFNNDIHHVRKEME VNFLSYVVLTVAAALPMLKQSN
SIVVVS
SLAGKIAHPLIAPYSASKFALDGFFSAIRKEHALTNVNVSITLCVLGLIDTD
TAMK
EVSGKIDMKAAPKEECALEIIKGGALRQDEVYYGNLQWTPLLLGNPGKRLIEFLHL
RKFDISKLVNN

Guinea pig (300 aa)

MAFLKKYLLT	ILMVFLAYYYY	YPANEKFRPE	MLQGKKVIVT
GASKGIGREI	AYHLAKMGAH	VVVVTARSKEA	LQKVVARCLE
LGAASAHYIA	GSMEDMTFAE	EFAAEAGNLM	GGLDMLILNH
VLYNRLTFFH	GEIDNVRKSM	EVNFHSFVVL	SVAAMPMLMQ
SQGSIAVVSS	VAGKITYPLI	APYSASKFAL	DGFFSTLRSE
FLVNKVNVS	TLCILGLIDT	ETAIKATSGI	YLGPAASPKEE
CALEIIKGT	LRQDEMYVVG	SRWVPYLLGN	PGRKIMEFLS
AAEYNWDNVL	SNEKLYGRWA		

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LDMLILNHVLYNRLTFFHGEIDNVRKSMEVNFSFVVL SVAAMPMLMQSQGSIAV
SSVAGKITYPLIAPYSASKFALDGFFSTLRSEFLVNKVNVSITLCILGLIDTETAI
KATSGIYLGPAASPKEECALEIIKGTALRQDEMYVVGSRWVPYLLGNPGRKIMEFLS
AAEYNWDNVLSNEKLYGRWA

Hamster (292 aa)

MHFMKKYLLP	ILVLFLAYYY	YSTKEEFRPE	MLQGKKVIVT
GASKGIGREM	AYHLSEMGH	VVLTARSEEG	LQKVASRCLE
LGAASAHYIA	GTMEDMTFAE	QFVLKAGKLM	GGLDMLILNH
ITYTSMNFFR	DEIHALRKAM	EVNFISYVVM	SVAALPMLKQ
SNGSIVVVSS	IAGKMAHPLV	ASYSASKFAL	DGFFSSLRRE
HGVTNVNVS	TLCVLGLINT	ETAMKATSGV	FNAPASPKEE
CALEIIKGGA	LRQEEVYYDS	WSWTPILLGN	PGRKIMEFLS
MKSFTFDKLI SS			

MHFMKKYLLPILVLFLAYYYYSTKEEFRPEMLQGKKVIVTGASKGIGREMAYHLSE
 MGAHVLTARSEEG LQKVASRCLELGAASAHYIAGTMEDMTFAEQFVLKAGKLMGG
 LDMLILNHITYTSMNFFRDEIHALRKAMEVNFISYVVM SVAALPMLKQSNGSIVVV
 SSIAGKMAHPLVASYSASKFALDGFFSSLRREHGVTNVNVSITLCVLGLINTETAM
 KATSGVFNAPASPKEECALEIIKGGALRQEEVYYDSWSWTPILLGNPGRKIMEFLS
 MKSFTFDKLISS

Rat (287 aa)

MKKYLLPVLV	LCLGYYYSTN	EEFRPEMLQG	KKVIVTGASK
GIGREMAYHL	SKMGHVVLT	ARSEEG LQKV	VSRCLELGAA
SAHYIAGTME	DMTFAERFVV	EAGKLLGGLD	MLILNHITQT
TMSLFHDDIH	SVRRSMEVNF	LSYVVLSTAA	LPMLKQSNGS
IAIISSMAGK	MTQPLIASYS	ASKFALDGFF	STIRKEHMT
KVNVSITLCV	LGFIDTETAL	KETSGIILSQ	AAPKQECAL
IKGTVLRKDE	VYYDKSPWTP	LLLGNPGRRI	MEFLSLRSYN
RDLFVSN			

MKKYLLPVLV LCLGYYYSTNEEFRPEMLQGKKVIVTGASKGIGREMAYHLSKMGH
 VVLTARSEEG LQKVVSRCLELGAASAHYIAGTMEDMTFAERFVVEAGKLLGGLDML
 ILNHITQT TMSLFHDDIHSVRRSMEVNFLSYVVLSTAALPMLKQSNGSIAIISSMA
 GKMTQPLIASYSASKFALDGFFSTIRKEHMTKVNVSITLCVLGFIDTETALKETS
 GIILSQAAPKQECAL EIKGTVLRKDEVYYDKSPWTP LLLGNPGRRIMEFLSLRSYN
 RDLFVSN

Mouse (292 aa)

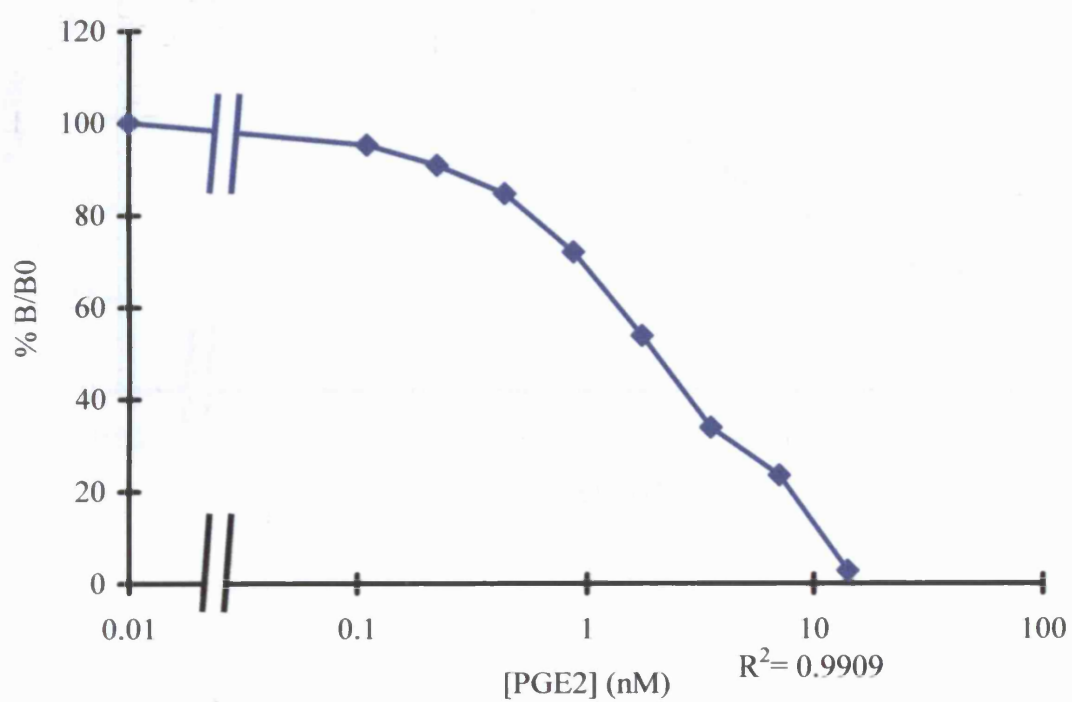
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ITQTSLSLFH	DDIHSVRRVM	EVNFLSYVVM	STAALPMLKQ
SNGSIAVISS	LAGKMTQPMI	APYSASKFAL	DGFFSTIRTE
LYITKVNVS	TLCVLGLIDT	ETAMKEISGI	INAQASPKEE
CALEIIKGTA	LRKSEVYYDK	SPLTPILLGN	PGRKIMEFFS
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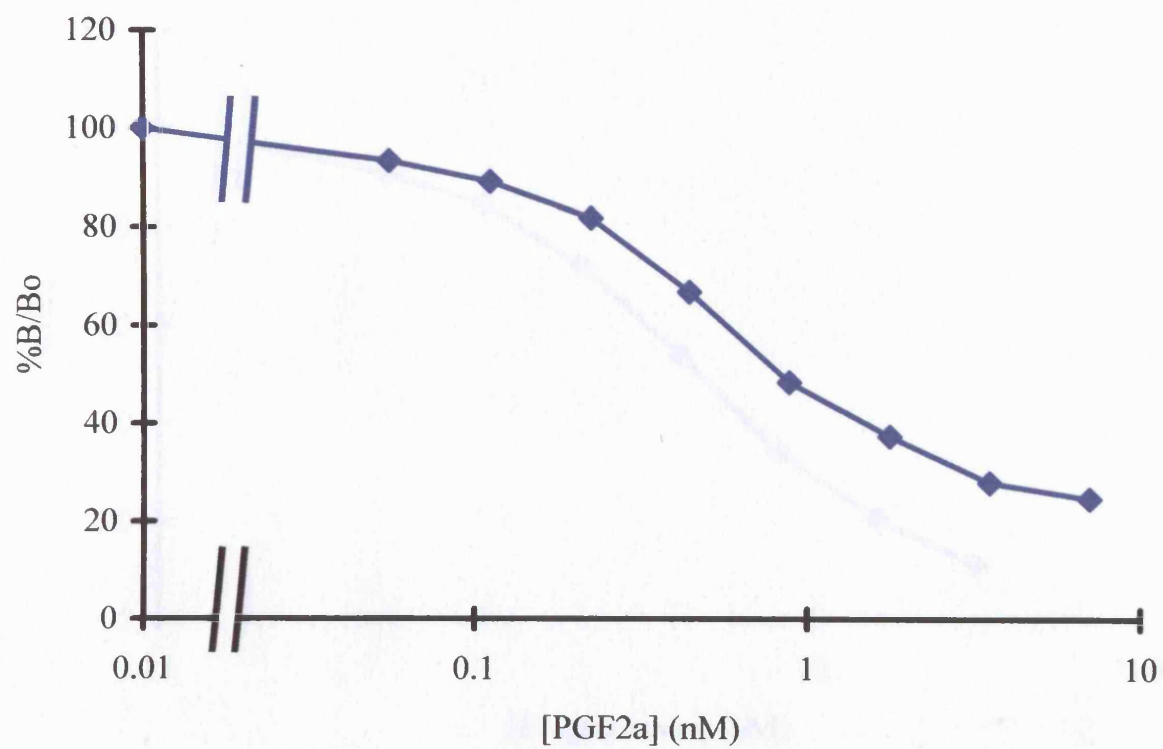
Appendix III

Appendix III

Standard curve for a PGE₂ radioimmunoassay

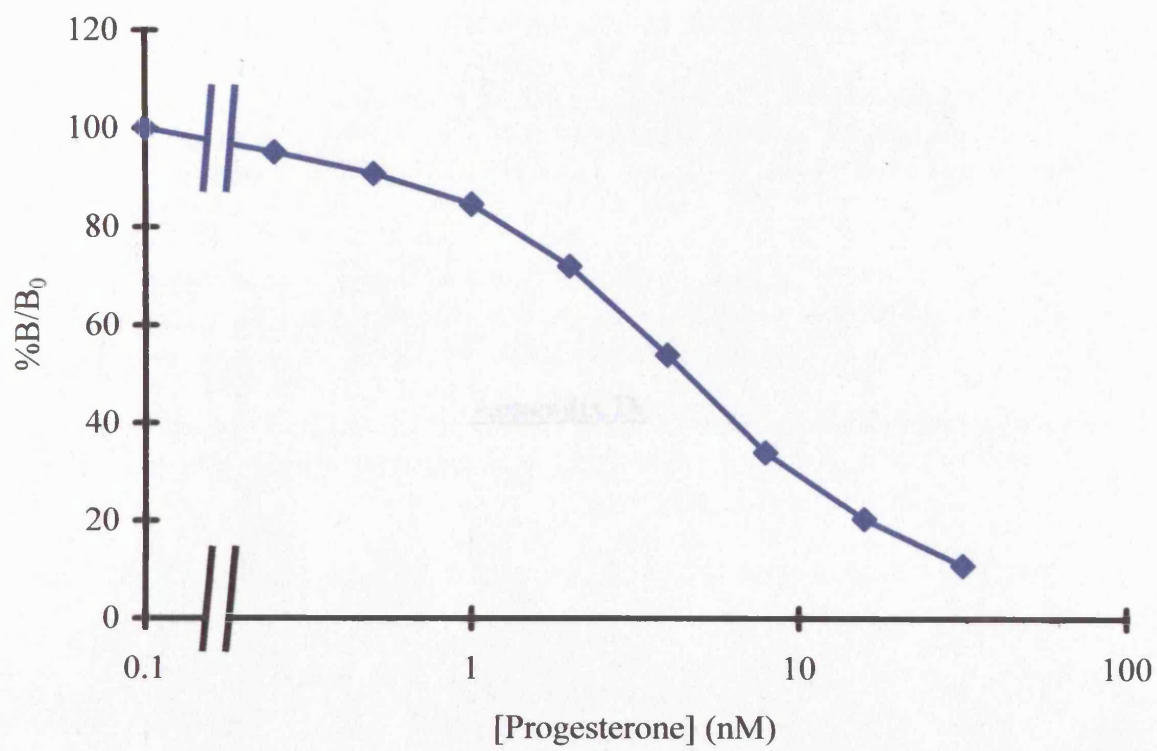


Standard curve for a PGF_{2α} radioimmunoassay



R²=0.983

Standard curve for a Progesterone radioimmunoassay



$$R^2 = 1.000$$

Appendix IV

Personal communication with Huang, received on 16/06/04:

Dear Dr. Kim Jonas,

I am very glad to hear from you.

Now I tell you some information about the gene 11-beta-HSD3.

This gene is homologous to HSD11B1 and HSD11B2. We have cloned this gene in human brain tissue designed SCDR10. its homologs in *Macaca fascicularis*, *Bos taurus*, *Gasterosteus aculeatus*, *Danio rerio*, *Gallus gallus* and *Oryzias latipes* were also cloned.

We have studied gene SCDR10 in detail. including northern blotting, protein expression and purification, enzyme activity assay, the mAb was also produced by immunizing rabbit and the immunohistochemistry analysis was also done.

I am very regretful I could not tell you the detailed result at present. Because we are writing a paper to report this gene. When our manuscript is accepted I will send you a copy in time.

If have chance we can cooperate. I am sure this gene is very important in human.

Best regards

Chaoqun Huang
Department of biochemistry
and molecular biology
Suzhou university